Papers

Beta$_2$-glycoprotein-1 (apolipoprotein H) excretion in chronic renal tubular disorders: Comparison with other protein markers of tubular malfunction

M Lapsley, P A Sansom, C T Marlow, F V Flynn, A G W Norden

Abstract
Urinary β_2-glycoprotein-1 was measured in 60 patients with conditions recognised as causing renal tubular impairment and compared with established markers of early tubular malfunction. Increased β_2-glycoprotein-1 excretion was found in 49 (82%) of the subjects; raised excretion of α_1-microglobulin, retinol-binding protein, and β_2-microglobulin was found in 46 (77%), 45 (75%), and 31 (52%), respectively, and increased urinary N-acetyl-β-D-glucosaminidase activity in 32 of 54 of the subjects (59%). The increase was particularly pronounced in those with proximal tubule malfunction, although considerable variation occurred.

β_2-glycoprotein-1 was shown to be stable in urine over the physiological pH range, and it is concluded that its measurement provides a means of detecting chronic malfunction of the renal tubules that is marginally more sensitive than assays of α_1-microglobulin or retinol-binding protein, and more reliable than assays of β_2-microglobulin or N-acetyl-β-D-glucosaminidase.

The early detection of proximal renal tubular malfunction depends on finding increased quantities of low molecular weight plasma proteins in the urine. These are freely filtered at the glomerulus and normally virtually completely reabsorbed by the proximal renal tubule cells, where they are catabolised. Defective reabsorption accounts for their increased urinary excretion in renal tubular disorders. β_2-microglobulin was the first of these proteins to be characterised and has been studied the most,[1] but because it is unstable in acid[2] and infected alkaline urine,[3] and may degrade during the time that the urine is held in the bladder,[4] its absence cannot be relied on to exclude the presence of a tubular proteinuria. Because retinol-binding protein and α_1-microglobulin are much more stable and not subject to increased production,[5 6] they are regarded as more reliable indicators of renal tubular malfunction.[7] In 1989 Donaldson et al claimed, however, that both can degrade significantly in non-alkalinised urine from patients,[8] although more recently Blumsohn et al concluded that immediate alkalinisation of urine was not

necessary for the measurement of retinol-binding protein.[9]

The urinary excretion of enzymes shed from proximal renal tubule cells has also often been used to detect tubular injury,[10] the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) frequently being chosen. Bernard et al, however,[11] found than NAG was much less sensitive that retinol-binding protein for the early detection of tubular impairment.

In the course of searching for improved markers for the recognition of tubular proteinuria, we discovered that β_2-glycoprotein-1 could be readily detected in the urine of patients with renal tubular disorders, and using a radial immunodiffusion assay we showed that it comprised a major urinary protein in such subjects.[12] The radial immunodiffusion assay lacked the sensitivity required for measurement of urinary β_2-glycoprotein-1 in healthy subjects and this led us to develop an enzyme linked immunosorbent assay (ELISA) capable of measuring concentrations down to 5 μg/l.[13] We used this more sensitive assay to investigate the stability of β_2-glycoprotein-1 in urine and to compare its excretion with that of currently recognised markers of tubular proteinuria in a spectrum of chronic renal tubular disorders. The study was approved by the hospital ethical committee.

Methods
Random specimens of urine were obtained from 60 patients unequivocally diagnosed as having conditions known to cause tubular proteinuria.[14] The patients comprised 31 males and 29 females, aged between 13 and 79 years, and included: 12 with Wilson's disease; 10 with familial distal renal tubular acidosis; seven with Dent's syndrome;[15] six with immune mediated distal renal tubular acidosis; six with chronic interstitial nephritis; five with renal allografts of between eight and 48 months' duration; three with inherited Fanconi's syndrome; three who had nephrocalcinosis associated with earlier hyperparathyroidism; three with medullary sponge kidney; two with analgesic nephropathy; one with the oculocerebro-renal syndrome of Lowe; one with chronic cadmium poisoning; and one with oxalate nephropathy following jejunal bypass surgery for gross obesity. Thirty two had plasma creatinine concentrations below 126 μmol/l, 17 concentrations between 126 and 175 μmol/l, and 11 concentrations exceeding 175 μmol/l.

Department of
Chemical Pathology,
University College
and Middlesex School
of Medicine,
Windeyer Building,
Cleveland Street,
London W1P 6DB
M Lapsley,
P A Sansom,
C T Marlow,
F V Flynn
Department of
Chemical Pathology,
Enfield District
Hospital, Middlesex
A G W Norden
Correspondence to:
Dr M Lapsley

Figure 1 Plots showing the effect of storage conditions on the stability of β_2-glycoprotein-1 in a urine specimen containing 86 $\mu g/l$. □—□ Aliquots adjusted to different pH values and kept for 18 hours at 4°C; O- - -O aliquots adjusted to different pH values and kept for 18 hours at 25°C.
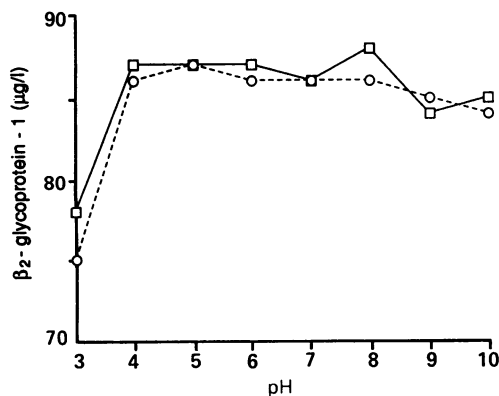
Single random specimens of urine were obtained from 35 of the patients, and between two and seven from the others, the total number of specimens available for the study being 103. Each urine specimen was centrifuged at 1800 × g for 10 minutes and the supernatant divided into portions for the different assays. All fractions were frozen within four hours of collection and were kept at −20°C until shortly before analysis.

All the urine samples were assayed for creatinine, albumin, β_2-glycoprotein-1, retinol-binding protein, α_1-microglobulin and β_2-microglobulin, and all but six for NAG. All the assays, except those for creatinine, were perfomed in duplicate, and control specimens were always included. To reduce the effects of the different concentrations of the urine specimens, the excretion of the proteins was related to that of creatinine, and to make comparison between the different proteins easier, all results were expressed in mg per mmol of creatinine. When several specimens were available from a subject, the average of all the results for a particular constituent was calculated.

The concentration of creatinine was measured by the Jaffé reaction, with the kinetic method used in the American Monitor Perspective analyser. Albumin was measured by an immunoturbidimetric method with a Cobas-Bio centrifugal analyser, using the Ames Microalb kit from Miles Laboratories Ltd (Stoke Poges, Slough, England). β_2-glycoprotein-1 was measured by an in-house sandwich ELISA technique,[13] retinol-binding protein by the ELISA method of Topping et al,[16] and α_1-microglobulin by the ELISA procedure of Takagi et al[17] using the Imzyne α_1M kit form the Mast Diagnostics (Bootle, Merseyside, England). β_2-microglobulin was measured by a radioimmunoassay method using the β_2-Micro RIA kit from Pharmacia Ltd (Midsummer Boulevard, Milton Keynes, England). NAG activity was measured by a

colorimetric procedure, based on the method of Yuen et al,[18] using a Cobas-Bio centrifugal analyser and the kit available from Cortecs Diagnostics Ltd (Deeside Industrial Park, Clwyd, Wales).

To investigate the stability of β_2-glycoprotein-1 in urine, portions of four specimens containing 86, 360, 1700 and 53000 $\mu g/l$ were adjusted to pH values between 3·0 and 10·0 by adding molar hydrochloric acid or molar sodium hydroxide. Sets of aliquots from three of the specimens were stored at 4°C and at 25°C for 18 hours, while a set from the fourth was incubated at 37°C for four hours. The β_2-glycoprotein-1 concentration of each aliquot was assayed after adjustment of the pH to 7·0, changes in volume attributable to the pH adjustment being noted and allowed for when calculating the results.

Results

The effect of varying pH on the stability of β_2-glycoprotein-1 in urine is shown in fig 1. This shows that β_2-glycoprotein-1 is stable between pH values of 4·0 and 8·0 for at least 18 hours at both 4°C and 25°C. Similar findings were obtained on the two other urine specimens stored at the same temperatures, and on the specimen incubated for four hours at 37°C no decline in concentration was observed at pH values between 5·0 and 8·0.

The mean and range of results obtained on the patients are given in the table, which also indicates the frequency and magnitude of increased excretion for each constituent, and quotes our reference ranges which were determined on 35 healthy adults. A wide scatter of values was obtained and increases in β_2-glycoprotein-1, retinol-binding protein, and α_1-microglobulin were found in about three quarters of the subjects, while measurements of β_2-microglobulin and NAG more often fell within the reference range. When an increased excretion was present, the increase was greatest in retinol-binding protein, and decreased in the order β_2-microglobulin > β_2-glycoprotein-1 > α_1-microglobulin > NAG.

The diagrams in fig 2 show the results obtained on the individual patients, grouped according to diagnosis, expressed as multiples of the upper reference limit and plotted on logarithmic scales. These show that there was considerable variation in the excretion of each constituent measured, even among patients with the same diagnosis. Increased excretion of β_2-glycoprotein-1, retinol-binding protein, α_1-microglobulin and β_2-microglobulin is particularly impressive among those with Lowe's syndrome, Fanconi's syndrome, and Dent's

Table Urinary excretion of specific plasma proteins and of NAG among patients with conditions associated with renal tubular malfunction

Urinary Constituent	Mean value	Range of results	Reference range	Patients with increased excretion Number (%)	Average increase
Albumin	26·0	0–156	<0·9	52 of 60 (87)	37 × Upper reference limit
β_2-glycoprotein-1	1·6	0·011–31	0·0069–0·0345	49 of 60 (82)	57 × Upper reference limit
α_1-microglobulin	6·1	0·1–63	<0·7	46 of 60 (77)	11 × Upper reference limit
Retinol-binding protein	5·5	0·003–184	0·001–0·016	45 of 60 (75)	455 × Upper reference limit
β_2-microglobulin	4·2	0·01–101	<0·1	31 of 60 (52)	81 × Upper reference limit
N-acetyl-β-D-glucosaminidase	40·6	2–189	<25	32 of 54 (59)	2 × Upper reference limit

Protein values are in mg and enzyme activity in μmol of substrate hydrolysed per hour, and all results are expressed per mmol of creatinine excreted.

Figure 2 Urinary excretion of specific plasma proteins and of N-acetyl-β-D-glucosaminidase, expressed as multiples of the upper reference limit, by patients with (1) Lowe's syndrome [□], chronic cadmium poisoning [▲], and oxalate nephropathy [o], (2) Fanconi's syndrome, (3) Dent's syndrome, (4) renal allografts, (5) chronic interstitial nephritis, (6) nephrocalcinosis complicating primary hyperparathyroidism, (7) analgesic nephropathy, (8) familial distal renal tubular acidosis, (9) Wilson's disease, (10) medullary sponge kidney, (11) immune-mediated distal renal tubular acidosis.
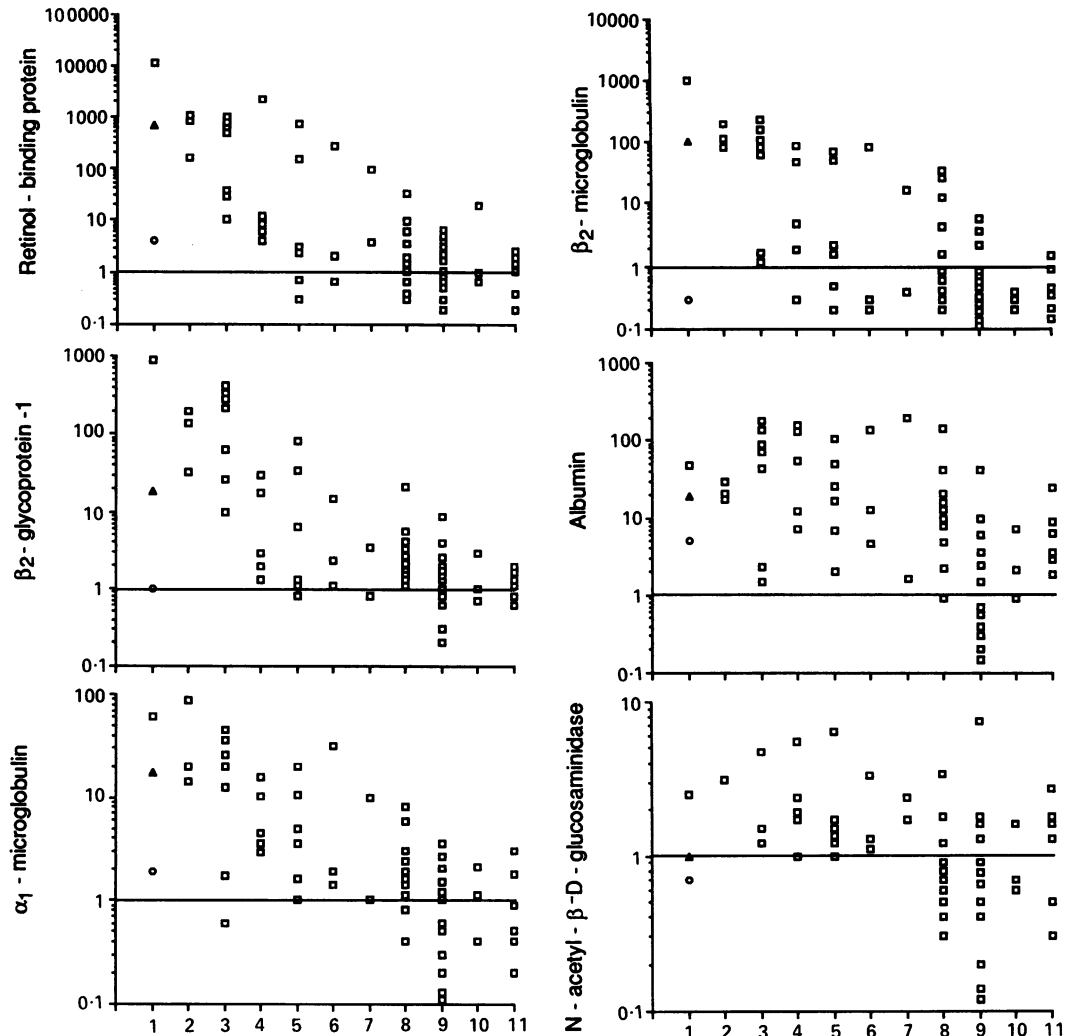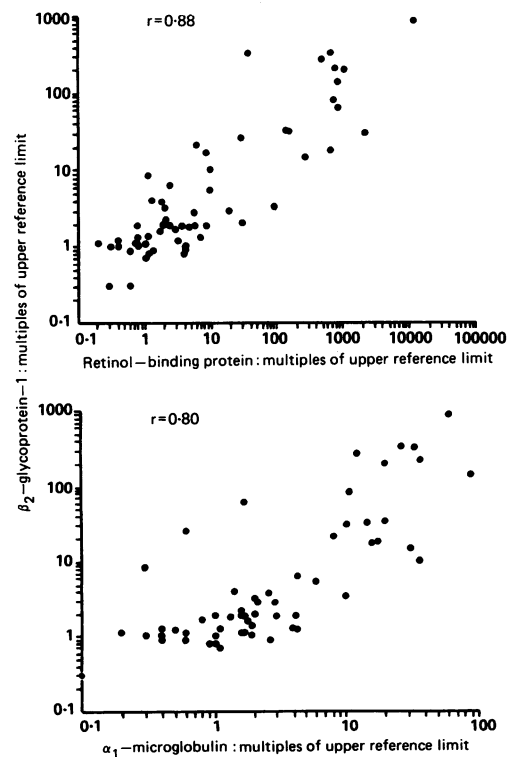

Figure 3 Plots showing the correlation between the urinary excretion of β2-glycoprotein-1 with that of retinol-binding protein and α1-microglobulin, among 60 patients with conditions known to cause chronic renal tubular malfunction. Excretion levels are expressed in multiples of the upper reference limit and plotted on logarithmic scales.

syndrome, but is also notable in those with chronic cadmium poisoning, renal allografts, chronic interstitial nephritis and nephrocalcinosis associated with previous hyperparathyroidism, all conditions known to be associated with malfunction of the proximal part of the renal tubule. By contrast, increased albumin and NAG excretion seem to vary within much the same limits in nearly all the disorders. Normal or near normal levels of excretion of all the constituents measured occurred particularly in the patients with Wilson's disease, familial renal tubular acidosis, immune renal tubular acidosis and medullary sponge kidney.

Generally, the results showed a close correlation between the amounts of β_2-glycoprotein-1, retinol-binding protein, and α_1-microglobulin excreted (fig 3), and this cannot be explained by the variation in the concentration of the urine specimens. Nevertheless, there was considerable variation in the patterns of urinary protein excretion among the patients. Comparison of the excretion of β_2-glycoprotein-1, retinol-binding protein, and α_1-microglobulin among all 60 patients showed concordance of the results in 68%, the excretion of all three proteins being increased in 37, and within the reference limits in four who had been treated for Wilson's disease. Discrepancies between the normality or otherwise of the excretion of
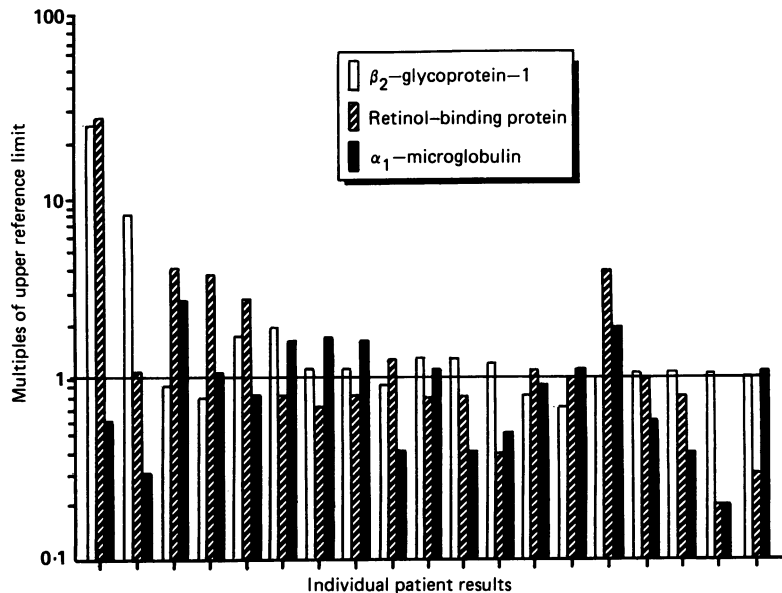
Figure 4 Diagram showing the excretion of β_2-glycoprotein-1, retinol-binding protein, and α_1-microglobulin by the 19 patients who had discordant results in terms of whether or not they fell within the reference range. All results are expressed as multiples of the upper reference limit.

β_2-glycoprotein-1, retinol-binding protein, and α_1-microglobulin were observed among the remaining 19 patients (fig 4); in five of these the discrepant result was very close to the upper reference limit, and the discordance probably reflected no more than the limitations of using statistically derived reference ranges to define normality.

Discussion

We previously reported that β_2-glycoprotein-1 was only present in the urine of patients with pronounced tubular proteinuria.[12] Using the more sensitive ELISA method,[13] we have now shown that 82% of those with conditions liable to cause tubular proteinuria were excreting increased quantities of β_2-glycoprotein-1, and the remaining 18% amounts that were close to the upper reference limit. The fact that we did not find increased excretion of this protein in all 60 patients does not necessarily imply that the assay lacks sensitivity for the detection of tubular malfunction; rather, it probably reflects lack of impairment of proximal tubular function among some of the patients. For instance, of the 12 patients with Wilson's disease, 11 had been treated with chelating agents to remove copper from their tissues for some years and tubular damage may either have never developed or have fully recovered. The patient whose urine was examined before chelation treatment started was the only one to show increased excretion of all the constituents measured.

Patients with conditions known to be associated with malfunction of the proximal part of the renal tubule were found to be excreting the largest amount of β_2-glycoprotein-1, which suggests that it is normally reabsorbed at this site. Most patients with distal tubular disease, however, were also found to be excreting increased quantities of β_2-glycoprotein-1 alongside retinol-binding

protein and α_1-microglobulin, although the amounts were much smaller. It may well be that because of the intimate nature of the blood supply to the different parts of the nephron that there is no such thing as pure proximal or distal tubular malfunction; the eventual development of impaired glomerular filtration in patients with Fanconi's syndrome and Dent's syndrome fits in with the concept that regardless of where the pathological process starts it is liable to spread to affect other parts of the nephron.

Comparison of the excretion of β_2-glycoprotein-1 with that of retinol-binding protein, α_1-microglobulin, β_2-microglobulin and NAG suggests that β_2-glycoprotein-1 is a promising marker of renal tubular malfunction and confers some advantage. Thus four patients showed increased excretion of β_2-glycoprotein-1 without increases in retinol-binding protein, α_1-microglobulin, β_2-microglobulin or NAG, although in two the increase was very marginal; only individual patients had lone increases of retinol-binding protein or α_1-microglobulin. The ability of β_2-glycoprotein-1 measurements to detect tubular malfunction among our 60 patients was, however, only slightly greater than that of retinol-binding protein and α_1-microglobulin—82% compared with 75% and 77%, respectively. The better performance of β_2-glycoprotein-1 measurements when compared with NAG assays, only 59% of which proved abnormal, may reflect the relative inactivity of the pathological process in the proximal tubule cells in many of the patients examined, because renal tissue proteins seem to be released near to the time of active cellular injury, whereas tubular proteinuria persists long after the causative agent has been removed.[19] The greater efficacy of the β_2-glycoprotein-1 assay over that of β_2-microglobulin was expected because it was not practicable to give oral sodium bicarbonate to the patients before collecting the specimens,[1] and we have shown that unlike urinary β_2-microglobulin, β_2-glycoprotein-1 is stable over a pH range of 4·0 to 8·0 for at least 18 hours.

An increased urinary excretion of β_2-glycoprotein-1 is not specific for renal tubular pathology because we have also found increases in patients with predominantly glomerular damage, as in the nephrotic syndrome or chronic renal failure.[20] In these cases β_2-glycoprotein-1, together with increased amounts of retinol-binding protein, α_1-microglobulin, and β_2-microglobulin, is excreted alongside considerable quantities of proteins of higher molecular weight, such as albumin and transferrin. In the nephrotic syndrome the increased excretion of low molecular weight plasma protein is probably explained by accompanying tubular malfunction, but in renal failure a major factor is the overwhelming of the reabsorptive capacity in the less affected nephrons by the increased filtered load, consequent on the raised plasma concentrations of these proteins. Virtually all of our subjects who were excreting increased quantities of low molecular weight proteins were also excreting increased amounts of albumin, so an increased

albumin excretion does not necessarily indicate glomerular disease.

Knowledge of the plasma creatinine concentration seems to be helpful when attempting to distinguish increased low molecular weight protein excretion due to tubular damage from that secondary to glomerular impairment. Bernard et al suggested that increased urinary excretion of retinol-binding protein could be regarded as diagnostic of impairment of the proximal renal tubule if the plasma creatinine was less than 177 μmol/l,[21] and Yu et al suggested the same in respect of α_1-microglobulin if the plasma creatinine was below 200 μmol/l.[6] One of our subjects with a normal excretion of retinol-binding protein had a plasma creatinine concentration of 171 μmol/l, confirming the view that a considerable reduction of the glomerular filtration rate is required to raise the plasma concentration, and consequently the filtered load of retinol-binding protein in the less affected nephrons to a point at which the normal tubular resorptive capacity will be exceeded. As one of our patients with a normal excretion of β_2-glycoprotein-1 had a plasma creatinine concentration of 210 μmol/l, the finding of increased excretion of β_2-glycoprotein-1 can probably also be regarded as diagnostic of tubular malfunction in the absence of a considerable rise in the plasma creatinine.

β_2-glycoprotein-1 has a molecular weight of around 50 kilodaltons[22] and consequently might not be expected to be filtered at all readily by the normal glomerulus. It is therefore perhaps surprising that it should be excreted in considerably increased quantity by patients with renal tubular defects who lack evidence of increased glomerular permeability. In this connection we recently reported that β_2-glycoprotein-1 has a range of isoelectric points which are relatively high, and suggested that as a consequence it is likely that it will pass through the glomerulus more readily than would be expected from consideration of its molecular weight.[12]

The physiological role of β_2-glycoprotein-1 remains unknown but it has been identified as a constituent of chylomicrons and very low density and high density lipoproteins,[23] and it is known to activate lipoprotein lipase and is sometimes referred to as apolipoprotein H.[24] It also inhibits the contact activation system in blood coagulation and binds specifically to platelet membranes.[25] Disorders other than those affecting the kidney may in the future be shown to have a bearing on its renal excretion, should they cause an increase in the plasma concentration.

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