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Research Article

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A Study of Urinary Excretion of Parathyroid Hormone in Man

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ABSTRACT A procedure for bioassaying parathyroid hormone-like activity in human urine has been developed. 24-hr urine samples were concentrated with dry Sephadex G-25 and bioassayed in the young thyroparathyrocauterized mouse by the measurement of whole blood calcium. Recovery of biological activity and radioiodinated beef parathyroid hormone was over 80%. Normal subjects usually excreted less than 30 U (USP) of activity per day while 18 patients with proven primary hyperparathyroidism excreted a mean of 182 U/day (USP). The activity was not found in 7 patients with hypoparathyroidism or in 5 patients with carcinoma of the breast, but was present in 9 patients with uremia and in 5 with carcinoma of the lung and hypercalcemia.

INTRODUCTION

Reported bioassay procedures for the measurement of parathyroid hormone (PTH)-like activity in urine in man have been complicated by the use of rather nonspecific assays based on a phosphaturic response in the test animal (1, 2) or have required 6-day urine collections to provide sufficient hypercalcemic activity to be measured in the parathyroidectomized rat (3). These methods have all depended on a relatively cumbersome benzoic acid coprecipitation method for concentrating urinary PTH-like activity before assay. We have developed a simplified procedure for separating this activity from 24-hr urine samples and have bioassayed these concentrates by a recently described procedure in mice, based on the relatively specific prevention of fall of serum calcium after thyroparathyroid cautery (4). This report records the use of this procedure in a series of patients with hyperparathyroidism, renal disease, and cancer.

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METHODS

All patients, except some with cancer, were studied during admission to the Los Angeles County-University of Southern California Medical Center. Those with primary and secondary hyperparathyroidism were admitted to the Clinical Research Center of the Los Angeles County-University of Southern California Medical Center. Urine samples were collected for control studies for a period of 24 hr from ambulatory laboratory and hospital personnel, as well as from patients with nonrelated mild illnesses. No attempt was made to regulate their calcium intake before the urine collection. Samples were kept refrigerated without preservatives during the collection period. Patients admitted to the Clinical Research Center were given a diet containing 600 mg of phosphorus per m^2 per day for 3 days for measurement of the per cent of phosphorus reabsorbed by the renal tubule (%TRP)), according to the procedure of Bernstein, Yamahiro, and Reynolds (5). Serum calcium was measured by the method of Fales (6), phosphorus by standard autoanalyzer techniques, and urine calcium by flame absorption spectrophotometry (7).

Concentration procedure. 24-hr urine samples were concentrated shortly after collection or frozen for later processing. All materials were kept at approximately 5°C before and during the procedure. The amount of dry Sephadex G-25 (coarse) to be added to the 24-hr sample was calculated by dividing the total volume of the sample by the water regain value given with each batch of Sephadex (usually 2.5 ml/g) to absorb all but 30–60 ml of the volume. The pH of the urine was adjusted to greater than 10 by the addition of approximately 2–3 ml of 1 N sodium hydroxide solution (NaOH) just before adding the calculated amount of Sephadex by quick stirring. The final concentration was usually less than 0.05 N NaOH. The mixture was then allowed to stand in the cold for 30 min. After this, the dry mash was quickly drained through a Buchner funnel with suction and dilute

0.10 N hydrochloric acid was added immediately to bring the filtrate to a pH of 7. The mash was washed twice with 50 ml of ethanol. The filtrate was then lyophilized and kept in a freezer until the time of assay when it was restored to a volume of 10 ml with 5% glucose and water. The recovered Sephadex was washed and dried for future use.

Recovery studies. Recovery studies have been performed in aqueous solutions; solutions containing 1% added human or egg albumin; in urine samples from normal subjects with Lilly parathyroid extract; with purified beef PTH prepared by the method of Rasmussen, Sze, and Young (8); and with radioiodinated beef PTH prepared by the method of Hunter and Greenwood (9). Biological activity was measured by the mouse bioassay (4) and ^{131}I radioactivity was determined in a Packard Gamma Counter (model 410 A). Purity of the iodinated PTH was checked before and after these procedures by electrophoresis in a 0.01 M phosphate buffer to verify the absence of damaged material in the iodinated hormone.

Recovery experiments were performed in order to evaluate the effectiveness of each step in the procedure finally developed after the initial recovery studies. Lilly parathyroid extract, 50 U, and 100,000 counts ^{131}I PTH (specific activity 250–300) were added to five 500-ml samples of normal urine on separate occasions. The pH was adjusted to greater than 10 with 1 N NaOH (end normality of the solution was between 0.02 and 0.05). The expected filtrate volume was calculated as 50 ml and sufficient Sephadex G-25 was added to give this volume (G-25 water regain value was 2.5 ml/g). After 30 min in the cold, the filtrate was collected by suction. A sample of this was counted for radioactivity. 50 ml of cold, absolute ethanol was added from a wash bottle to the Sephadex in the Buchner funnel and the solution again collected by suction and called the first wash. The same procedure was followed for the second wash. The recovered counts were corrected at each stage for damage, which was less than 5%, by paper electrophoresis.

Biological assay procedure. The biological activity of the urine sample concentrates was compared to that of Lilly parathyroid extract by subcutaneous injection at 2-dose levels (usually 0.5 and 2.0 U/mouse) into two groups of 8–10 thyroparathyrocauterized 3-week old, 10-g mice for each patient. The lyophilized urine concentrate was dissolved in 10 ml of 5% glucose solution just before assay. All injections were given in a final volume of 0.4 ml/mouse by appropriate dilution in the glucose solution. After 5 hr, blood calcium was measured by flame absorption spectrophotometry in 100-μl aliquots of blood, obtained by decapitation. The assay was regularly sensitive to at least 0.5 U of the Lilly reference standard.

Statistical analysis. After the completion of each assay, a visual estimate of the sample potency was made by plotting the log-dose response of the sample and standard on graph paper. Later, as sufficient samples accumulated, the data was analyzed on a computer program based on the statistical method of Finney (4, 10). From this, an estimate of the 95% fiducial limits of the mean dose response was obtained as well as an estimate of the non-linearity of response. Unfortunately, the high dose of concentrate may kill sufficient animals to prevent analysis in this 4 point statistical system. In those instances, only an "estimate" was obtained and the fiducial limits were not recorded. The statistical design of this method is such that all groups of mouse calcium values for each point must contain equal numbers. In those cases where unequal numbers of mice survived, values were discarded for entry into the computer program but not for the visual "estimate" and, therefore, in some instances a discrepancy between the estimated potency and calculated potency may appear.

RESULTS

Recovery experiments (Table I). Recovery of both radioiodinated PTH and biological activity of parathyroid extract was consistently from 80 to slightly over 100% in the complete concentration procedure. Exposure of these materials to 0.05 N NaOH without extraction for comparable periods of time produced no loss of biological activity or accelerated decay of the radioiodinated material. If alkali was not added to the sample recovery could fall to as low as 15–25%. This was improved by the addition of 0.1% human or egg albumin to the sample before concentration, but not to the consistently high recoveries obtained when the urine was brought to a pH of 10. When this was done, the addition of albumin did not substantially increase recovery, which suggested that the routine addition of albumin to the urine before concentration did not seem warranted. Losses of radioiodinated PTH to the glassware were less than 1% in the presence of an alkaline sample. Most of the loss was accounted for by accumulation of material within the Sephadex and was very high in acid urine specimens. It could be greatly reduced by albumin addition to the sample in the acid state. The over-all recovery and

TABLE I

The Recovery of Beef ^{131}I PTH from Urine by Sephadex Concentration

Experiment	Per cent recovery of counts added			
	Filtrate	1st Wash	2nd Wash	Total
1	23.4	68.3	11.7	103.4
2	31.4	43.6	15.8	90.8
3	18.8	44.8	18.8	85.4
4	28.2	39.4	27.7	95.3
5	23.2	61.9	11.8	96.9

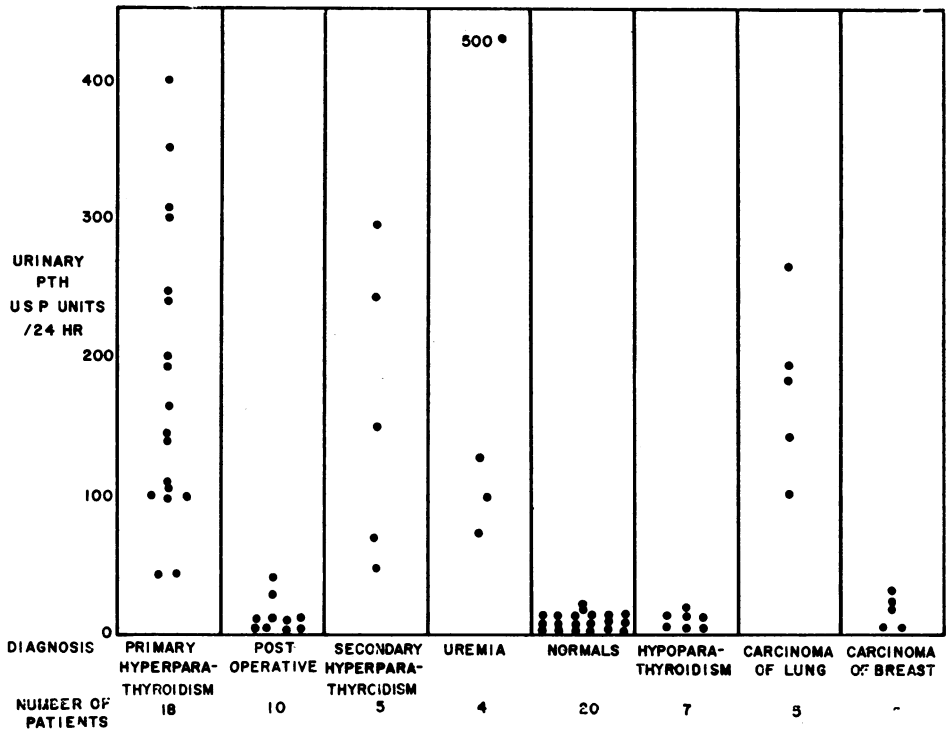

FIGURE 1 Results obtained with the concentration procedure and bioassay in several groups of patients with diseases in which a disturbance of serum calcium may occur. Each point represents the estimated value from a single assay result for each patient.

loss at each step as determined by ^{131}I PTH is shown in Table I.

Control subjects (Fig. 1). If more than 0.4–0.5 ml of the reconstituted lyophilized concentrate was injected into a mouse, a high mortality rate usually ensued. Since the usual lower dose of reference standard was 0.5 U, this represented a lower limit of sensitivity of 12–15 U/day on a 2 point sample curve, using the dose ratio of 1 : 4 for both the sample and reference standard. Most of the normal subjects had levels not detected by even the higher single dose of the sample and none had sufficient activity to give a valid 4 point assay analysis. Those urine PTH values in the normal subjects, shown to be above 15 U/day, were estimates based on the mouse calcium response on a single assay point (8–10 animals) which was numerically higher than the dose response of the lower Lilly parathyroid extract standard as estimated by simple plotting. No normal subject had repeatable values over 30 U/day. On a single assay, a normal subject occasionally gave a high value which on repeated determination of appropriate dilutions contained no activity.

Patients with parathyroid disease. The majority of patients with primary hyperparathyroidism excreted more than 100 U of PTH-like activity per day (Fig. 1). The estimated and calculated values of PTH, as well as the pertinent routine clinical laboratory data, are given in Table II. After operation in 10 of the 18 patients, no detectable activity was found except in two patients, in whom very slight activity was present only at the single high dose level of injection. In these two patients, the preoperative urinary PTH-like values were 400 and 95 U/day. No significant detectable activity was found in seven patients with hypoparathyroidism. Four of five patients with primary renal failure and radiological demonstrable bone disease clearly had PTH excretion rates in the range of those found in primary hyperparathyroidism without significant renal failure (Table III). One patient, a 6 yr old girl with secondary hyperparathyroidism, had a lower value with a mean PTH excretion of 50 U/day with 95% fiducial limits of 28–72, as demonstrable in a 4 point assay with acceptable linearity between the sample and standard PTH. This value may

TABLE II

Calculated and Estimated Urinary PTH-Like Activity and Pertinent Clinical Data in 18 Patients with Proven Primary Hyperparathyroidism

Patient	Parathyroid hormone: Calculated value Mean	Parathyroid hormone: Calculated value Range 95%	Parathyroid hormone: Estimated value	Serum: Calcium	Serum: Phosphorus	Urine calcium	TRP*
		U/24 hr		*mg/100 ml*		*mg/24 hr*	*%*
T. D.	346	230–463	348	11.1	2.6	165	54
M. O.	239	159–319	246	12.8	2.5	269	70
M. L.	142	76–208	167	12.6	2.1	193	71
O. L.	25	18–32	39	10.7	2.8	320	84
A. C.	32	5–59	39	12.7	1.9	486	73
M. K.	232	64–400	315	12.4	2.4	473	59
H. M.	204	138–270	189	11.4	2.6	214	70
H. L.	164	92–236	145	12.0	3.9	215	85
W. L.	—	—	200	12.9	2.5	330	68
L. M.	—	—	118	11.4	3.1	350	85
K. K.	—	—	400	11.3	2.5	90	53
E. S.	—	—	110	13.2	2.3	240	64
S. A.	—	—	100	11.4	2.5	392	87
A. L.	—	—	240	11.0	4.0	59	—
S. O.	—	—	300	12.9	1.7	332	85
R. M.	—	—	135	11.4	2.5	153	78
V. T.	—	—	95	12.2	1.9	608	74
P. H.	—	—	100	11.4	2.5	370	—

* Normal range = 86–96%, tubular reabsorption of phosphorus.

be considered elevated for her age and size but we have not assayed any subjects of similar age and size for comparison. Four patients with uremia and no radiological demonstrable bone changes also had elevated levels of PTH-like activity. Five patients with carcinoma of the lung and hypercalcemia had elevated levels of activity not differing from those with hyperparathyroidism. In one pa-

TABLE III

Calculated and Estimated Urinary PTH-Like Activity and Pertinent Clinical Data in 10 Patients with Primary Renal Disease

Patient	Parathyroid hormone: Calculated value Mean	Parathyroid hormone: Calculated value Range 95%	Parathyroid hormone: Estimated value	Serum: Calcium	Serum: Phosphorus	Urine calcium	Alk. P'tase. B.L. units	Blood urea nitrogen	Duration
		U/24 hr		*mg/100 ml*		*mg/24 hr*		*mg/100 ml*	*yr*
C. C.	149	16–282	150	10.2	10.1	72	18.0	141	9
A. O.	69	29–110	72	7.8	6.3	17	30.0	129	12
R. G.	237	113–361	256	7.3	9.5	80	6.5	282	2
E. D.	288	148–428	280	7.4	8.7	105	22.0	130	3
E. D.*	60	0–116	98	10.0	7.4	40	21.0	130	3
P. B.	50	28–72	51	5.4	15.6	28	14.0	140	4
J. V.	—	—	513	7.5	9.2	—	—	198	15
J. W.	—	—	70	7.4	10.3	62	2.0	220	15
G. D.	—	—	100	7.0	6.7	16	6.5	120	6
M. N.	—	—	130	7.5	7.6	11	—	162	2

B.L., Bessey-Lowry.

* Repeated values after treatment with vitamin D for several months.

tient with carcinoma of the lung and hypercalcemia, urinary PTH-like activity, and serum calcium concentrations returned to normal after treatment with methotrexate (*N*-[*p*-[(2,4-diamino-6-pteridinylmethyl)methylamino]benzoyl]-glutamic acid). Demonstrable bone lesions were not present in these patients. In five patients with carcinoma of the breast, three of whom had hypercalcemia, no activity greater than 37 U/day was found.

DISCUSSION

Previously reported methods (1–3) for the measurement of PTH-like activity in urine have all used the benzoic acid precipitation procedure based on a method used to extract PTH from ox glands (11). Recovery rates of Lilly parathyroid extract were recorded by all groups and ranged from 48 to 85% with a mean of 68%. Preliminary studies with this procedure in our laboratory, showed that it was very time consuming to perform and that the material obtained was toxic in our mouse assay preparation. Sephadex G-25 has the property of removing water and solutes of less than 5000 mol wt from solutions and excluding substances of larger molecular weight such as PTH. The dry Sephadex G-25 when added to urine removes most of the materials toxic to the thyroparathyroidectomized mouse. However, it was first found that very poor recovery rates were often obtained even with the addition of albumin to decrease the chance of surface absorption that occurs in Sephadex columns and on glassware. The removal of insufficient water from the Sephadex was considered to be the cause of these poor recoveries but washing the Sephadex slurry with ethanol to remove a certain portion of the withheld water did not greatly improve the per cent of PTH recovered. When the pH was adjusted to alkalinity uniform recoveries of over 80% were obtained with both the bioassay of added parathyroid extract and the addition of radioiodinated pure beef PTH. Since polypeptide bonds are unstable in alkali solution, the period of contact must be minimized by adjusting the pH of the urine just before the addition of the dry Sephadex and by neutralizing the concentrate as soon as possible after its rapid removal. However, in this procedure the final solution is never greater than 0.05 N NaOH and our studies, allowing the addition of alkali for periods of time greatly in excess of that using the concentration procedure, produced no loss of biological activity. With this procedure, one operator can process several urines per day. Washing and drying of the used Sephadex may take longer and appears to be the major disadvantage of this method for the concentration of PTH from urine.

Two of three publications recording the presence of PTH-like activity in urine used an assay based on the phosphaturic response to injected material in the intact mouse (1) and the ^{32}P-loaded parathyroidectomized rat (2). Their results have been criticized for the dubious specificity of the phosphaturic effect of crude extracts (12). The third report used a method based on the more specific hypercalcemic effect in the parathyroidectomized rat (3) but the assay as reported required large amounts of PTH for a statistically satisfactory assay and necessitated collection of urine for 6 days for reliable results. The assay used in our laboratory is based on this more specific hypercalcemic effect of PTH and is approximately 10 times as sensitive as the assay used by Eliel, Chanes, and Hawrylko (3), so that valid assays can be obtained with a 24 hr sample of urine or less if the sample content of PTH is very high.

Because of the small size of the mouse, greater care must be taken in administering extracts, withdrawing blood, and general performance of the assay to avoid greater assay error than is obtained with the rat procedure. This may lead to wide fiducial limits for the assay system and tends to limit the validity of a single measurement of PTH-like activity in urine to an estimate rather than a precise value. However, it does appear to separate patients with high urine PTH-like activity from those with low activity and as such is of considerable practical significance.

The values reported for patients with surgically proven primary hyperparathyroidism in this study are significantly higher ($P < 0.01$) than reported previously, although the number of patients to compare these values with is small. Davies (1) estimated the average daily urine output in four patients with primary hyperparathyroidism and renal insufficiency to be 121 PTH U/day with a range of 103–146, using the phosphaturic assay; Fujita, Morii, Ibayashi, Takahashi, and Okinaka (2) using the ^{32}P assay, reported a value of 65 PTH U/day with fiducial limits of 38–168 in one patient with hyperparathyroidism; and Eliel et al.

(3) recorded an average value of 94 PTH U/day with a range of 59–120 in three patients with primary hyperparathyroidism. The mean estimated value in the patients with primary hyperparathyroidism in our study was 182 PTH U/day with a mean range of 39–400.

PTH-like activity in 24-hr urine samples from normal subjects was similar in all groups: Davies reported a range of 42–70 U/24 hr, Fujita et al. 0–30 U/24 hr, Eliel et al. 0–37 U/24 hr, and in our assay 0–30 U/24 hr in 21 normal subjects. The reference standards, on which these comparisons over a 10 yr period have been made, were commercially prepared extracts standardized for clinical use. There is as yet no pure international standard PTH on which to make a comparison. Bioassay results obtained after surgical removal of the parathyroid adenoma in the patients with hyperparathyroidism and in those patients with hypoparathyroidism were as expected. Vitamin D and its urinary metabolites in the patient with hypoparathyroidism does not interfere with this bioassay (4).

No recorded results of PTH-like activity in patients with primary renal disease can be found in the literature. Elevated plasma PTH, as measured by radioimmunoassay, has been recently reported in a group of patients with chronic uremia (13). The values were frequently much higher in uremia than in adenomatous hyperparathyroidism and a relationship between the degree of parathyroid hyperplasia and the duration of the uremia was suggested. This was not evident in the urinary PTH-like activity in the small series of patients herein reported, although the urine values were very high in view of the greatly reduced renal glomerular filtration rate that was present. No information is available on the effect of a decreasing glomerular filtration rate on the renal clearance of PTH. In the urine of two normal subjects, Eliel et al. reported the recovery of 18 and 19% of 400 U of parathyroid extract given intramuscularly. Three of the four patients with primary hyperparathyroidism, in which urine values were recorded by Davies, had very severe renal disease and may, as she suggested, have produced lower urinary values for PTH as compared to patients with hyperparathyroidism and normal renal function. Regardless of this lack of knowledge of the renal clearance of PTH in uremia, the mean excretion rate of 180 PTH U/day in the nine patients was not different from the larger group of patients with primary hyperparathyroidism and normal renal function whose mean excretion rate was 182 PTH U/day. This suggests, in agreement with the results of the immunoassay, that PTH levels may be very high in secondary hyperparathyroidism as compared to primary hyperparathyroidism. Five of the nine patients had obvious renal osteodystrophy. In one of these patients, treated with 200,000 U of vitamin D, the serum calcium rose to a high normal level, the serum alkaline phosphatase decreased, the X-ray evidence of osteodystrophy decreased, and the urinary PTH-like activity fell from a mean of 288 to 60 with clearly separable fiducial limits. Berson and Yalow (13) demonstrated a rapid fall in plasma radioimmunoassayable activity with the infusion of calcium into two patients with uremia and elevated serum PTH.

Considereable evidence has accumulated to suggest that nonparathyroid tumors, especially those of the lung, may produce a parathyroid-like substance (14). This has been shown to be present in plasma by radioimmunoassay (13, 15), in the tumors by immunologic techniques (15, 16), but not by any reported bioassay procedure. PTH-like activity has been reported to be distinctly elevated (107 U/day) in the urine of one patient with hypercalcemia and lung carcinoma by Eliel et al. (3). The five patients in our study with lung cancer and hypercalcemia had no demonstrable metastases and all had elevated PTH-like activity in the urine. In one patient, the elevated urinary concentration returned to normal after a reduction of the serum calcium to normal levels with methotrexate therapy.

This biologically active substance found in urine is hypercalcemic and phosphaturic in rats and mice, occurs in situations where it might be expected to be found, and is absent when it might reasonably be expected to be so. Eliel et al. have shown that the excretion rate of the material can be increased by giving the patient a low calcium diet. In one patient with uremia in our study, raising the serum calcium by vitamin D therapy resulted in a lower urinary excretion of PTH-like activity. Thus, the material seems to behave in a manner similar to biologically active PTH and to its normal physiological stimulants. The occurrence of such a polypeptide in urine is not unique. Gonadotrophic hormones with molecular weights in excess of 20,000 have been routinely measured

in urine for years (17). Insulin has been found in urine (18) and both adrenocorticotropic hormone (19) and growth hormone (20) have been reported as present in urine in small quantities.

Both bioassayable (21, 22) and immunoassayable (12, 23) PTH has been found in human plasma or serum. The concentration of bioassayable plasma PTH as measured by a phosphaturic assay with 50% recovery rate has been reported to be at least 10 times as high as the concentrations previously found in urine (22). Ready correlation of urine values with the immunoassayable levels in the plasma is difficult because of the lack of a precise estimate for the absolute concentration of PTH in human plasma (due to poor cross-reactivity between human and beef PTH), the lack of a suitable standard preparation of human PTH (12), and more precise knowledge of the distribution volume and decay rate of PTH in man. The ease and relative cheapness of this reported procedure may make it suitable as an aid in the differential diagnosis and for physiological studies in patients with various parathyroid disorders.

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