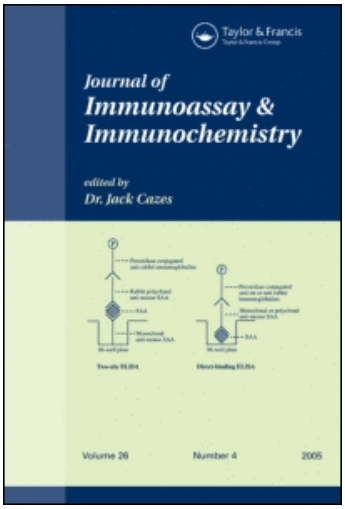

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A RADIOIMMUNOASSAY TO MEASURE PIRETANIDE IN HUMAN SERUM AND URINE

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ABSTRACT

This paper describes a specific radioimmunological method for determining the diuretic agent piretanide (4 phenoxy-3-(1-pyrrolidinyl)-5-sulfamoyl benzoic acid) in human serum, plasma and urine.

The antiserum was raised in rabbits against an immunogen of piretanide coupled to bovine serum albumin. The iodinated hydroxy derivative of piretanide was used as tracer. The separation of free and antibody-bound piretanide was performed by precipitating the antibody-tracer complex by polyethylene glycol.

The limit of detection was 4 ng/ml. Studies on specificity showed less than 0.5 % cross-reactivity of an identified metabolite. Intraassay reproducibility showed an average coefficient of variation of 7.3 %, the interassay variation was 5 %. A recovery experiment yielded 100.9 % recovery. There is good agreement between parallel determinations of piretanide by RIA and HPLC in both human serum and urine.

INTRODUCTION

Piretanide (HOE 118, Arelix(R), (4 phenoxy-3-(1-pyrrolidinyl)-5-sulfamoyl benzoic acid), (Fig. 1) is a potent new diuretic agent. Details on synthesis and animal pharmacology have been published recently (1). In man, 6 mg piretanide are equipotent

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with 40 mg Lasix (2). In healthy subjects only unchanged piretanide but no metabolites could be detected in blood (3). In human urine five metabolites have been separated by TLC and one of these has been identified (Fig. 1).

The low oral doses and rapid elimination of the drug require a sensitive method for monitoring concentrations in blood and urine.

This paper describes a sensitive and specific radioimmunoassay for determination of piretanide in serum, plasma or urine. Validation was performed according to the recommendation of the International Federation of Clinical Chemistry (4).

MATERIAL AND METHODS

Preparation of the Immunogen

Piretanide was coupled to bovine serum albumin (BSA), (Behringwerke Marburg, FRG), by a methylene bridge, using a MANNICH reaction (5). Piretanide was first treated with formaldehyde under acidic conditions, yielding the hydroxymethylated sulfonamide derivative. This was added to a BSA solution at pH 8.5 - 9.0. After reacting for 16 hours at room temperature, the piretanide-BSA-conjugate was purified by ultrafiltration using a PM 30 membrane filter (Amicon Corp. Lexington/Mass. 02173, USA). The product was lyophilized and then dried over P_2O_5. The piretanide/BSA ratio was determined by UV absorption at 345 nm in 1 % $NaHCO_3$ solution.

Production of Antiserum in Rabbits

Four white New Zealand rabbits weighing 3 - 4.5 kg were immunized with the piretanide-BSA-conjugate, using the same procedure for all animals. The immunization solution contained 0.75 mg piretanide conjugate emulsified in 2 ml saline solution plus 2 ml Freund's complete adjuvant. This solution was injected subcutaneously at 8 different sites in the region of lymph nodes. From the 2nd to the 5th day the immunization was continued by daily intravenous injection of 0.1 mg conjugate dissolved in 3 ml 0.05 % aerosil solution. The whole procedure was repeated three and five weeks after the start of the immunization. Each rabbit received a total of 3.45 mg piretanide-BSA-conjugate. The first blood sample was taken 36 days after the start of immunization. The serum contained high concentrations of antibodies to both bovine serum albumin and piretanide-BSA-conjugate, detected by immunoelectrophoresis. A similar injection sequence was given as a booster in the 7th week. The production of antibodies against piretanide was measured by the standard RIA procedure using 1 µl of antiserum, and tracer equivalent to 20,000 cpm in a total volume of 0.4 ml. From the 8th week after the start of immunization, 50 ml blood samples were taken 3 - 4 times per week. The antiserum, obtained by natural coagulation of the blood was collected and pooled after determination of the antibody titre.

A pooled serum from one rabbit was used for RIA development and for all routine assays. One ml portions of antiserum, sufficient for 10,000 single determinations, were stored at -70°C.

The material has been stable since 1978, and after thawing, aliquoting and refreezing is stable for at least 1 year.

Preparation of Tracer

Piretanide itself cannot be directly iodinated. The hydroxy derivative 4(4-hydroxy-phenoxy)-3-pyrrolidinyl-5-sulfamoyl-benzoic acid (Fig. 1) was therefore synthesized. This compound was iodinated in phosphate buffer by the chloramine-T method (6). Purification of the iodinated product was unnecessary, since the iodination yield exceeded 95 %. The labelled product had a specific radio-activity of 150 - 200 mCi/mg. The shelf-life of the tracer was at least 4 weeks in the lyophilized state. Damage was indicated by lower zero-binding and an increase in non specific binding.

Standard Material

Piretanide solution salt, source Batch no. Op 17a, was used as standard. The material contained less than 0.1 % immunoreactive impurities as shown by HPLC separation (7). Stock solutions of 0.1 mg piretanide per ml were stored in 0.5 ml aliquots at -20°C.

Other Materials

All chemicals were of analytical reagent quality. PEG 6000 was purchased from Riedel de Häen, Darmstadt, FRG; bovine serum albumin from Behringwerke Marburg, FRG; human serum and plasma

were prepared from the blood of healthy volunteers after an overnight fast and 20 ml aliquots were stored at -20°C.

Buffer and Working Dilutions

For all experiments we used phosphate buffer 10 mmol/l, pH 7.4 containing additional 8.77 g NaCl, 1 g bovine serum albumin and 1.0 g NaN_3 per litre. This solution is stable for 1 month at 4°C.

Standard buffer was used for dilution of antiserum (1 µl/ml), tracer (200 000 - 250 000 cpm/ml), standards (200, 100, 50, 25, 12.5, 6.3, 3.2, 1.6 ng/ml), and interassay control solutions (45, 350, 775 ng/ml).

Specimen Collection and Handling

0.5 ml aliquots of heparinized plasma (10 U heparin per ml blood), 0.5 ml serum or 1 ml urine were deep-frozen within 30 minutes of collection and remained stable for 2 years.

Experiments to Establish Optimum Assay Conditions

The concentration of piretanide in plasma after oral administration of 6 mg tablets lies within the range of 5 - 400 ng/ml plasma. Using 20,000 cpm tracer (approx. 20 pg), 50 % binding can be achieved when 0.1 µl antiserum is added to each tube in a total volume of 0.35 ml. The assay conditions were adjusted for determination in 50 µl plasma. No difference was detected between the standard curve prepared in serum or heparinized plasma. In cases of higher piretanide concentration smaller volumes of plas-

ma must be used and made up to 50 µl with piretanide-free human plasma. The presence of 50 µl plasma or serum in standard and all unknown samples (Tab. 1) is necessary in order to guarantee optimal separation of antibody-bound and free tracer by PEG precipitation.

There was an increase in the precipitated antibody-antigen-complex up to 200 g PEG/l buffer when 1 ml solution was added to each tube. Using higher concentrations of PEG, the precipitation rate remained constant. In the standard assay 230 g PEG/l were used, resulting in optimal separation of antibody-bound tracer and the unbound fraction. No time-dependent influence on tracer precipitation could be detected when the mixture was left to stand for up to 20 minutes.

To investigate the kinetics of tracer-binding to the antibody, incubations were performed for various times up to 24 hours at room temperature and at 4°C. After 4 hours, no increase in the amount of tracer bound to antibody was observed. At 4°C the tracer binding is higher than at room temperature. The experiments favour an incubation at 4°C and incubation times between 5 and 24 hours.

Assay Performance

All determinations were carried out in triplicate in polystyrene tubes (12 x 55 mm). Assay tubes were set up as shown in Tab. 1 and the tubes were incubated overnight at 4°C. After addition of 1 ml PEG solution the tubes were mixed with a Whirly-

TABLE 1

Assay Flow Sheet for Piretanide-RIA. Volumes are given in ml/tube.

	Human Plasma	Buffer	Standard Dilution	Tracer Dilution	Antiserum Dilution
Total Counts	0.05	0.2	-	0.1	-
Non-specific Binding	0.05	0.2	-	0.1	-
Zero-Binding	0.05	0.1	-	0.1	0.1
Standard	0.05	-	0.1	0.1	0.1
Controls	0.05	Control Solutions 0.1	-	0.1	0.1
Plasma Unknown	0.05	0.1	-	0.1	0.1
Urine Unknown	0.05	0.05 Urine 0.05	-	0.1	0.1

mixer, left to stand for 10 min. and then centrifuged at 2000 g for 15 min. The supernatant was decanted and the tubes inverted on absorbent cellulose. The radioactivity in the precipitate was determined in a well-type gamma-counter.

Calculation of Drug Concentrations

The concentrations of piretanide are read from the standard curve using a non-linear spline approximation (8).

RESULTS

Specificity

The specificity of the assay for piretanide was investigated by incubating increasing concentrations of the identified metabolite (Fig. 1) with the antibody. Comparing the concentrations at a 50 % tracer displacement (Fig. 2), the cross-reaction of the metabolite is less than 0.5 %. No cross-reactivity with commercially available diuretics, (chlorthalidone, bumetanide, hydrochlorothiazide, amiloride, ethacrynic acid, triamterene, furosemide) could be detected when added at up to 10 µg per tube. No effect of other drugs has been identified in samples from patients receiving multiple drugs.

Limit of Detection

A typical standard curve is shown in Fig. 2. The mean and standard deviation of the radioactivity of a blank (zero-standard) were determined in triplicate for each standard curve. The concentration corresponding to 3 standard deviations was taken as the detection limit. During routine determination over 2 years this value ranged from 1.0 - 4.0 ng/ml, therefore the detection limit of the assay was set uniformly at 4.0 ng/ml.

Reproducibility

Piretanide was added to human serum at concentrations ranging from 6 - 10 000 ng per ml in order to test intraassay imprecision. The resulting samples were divided into 10 aliquots and piretanide

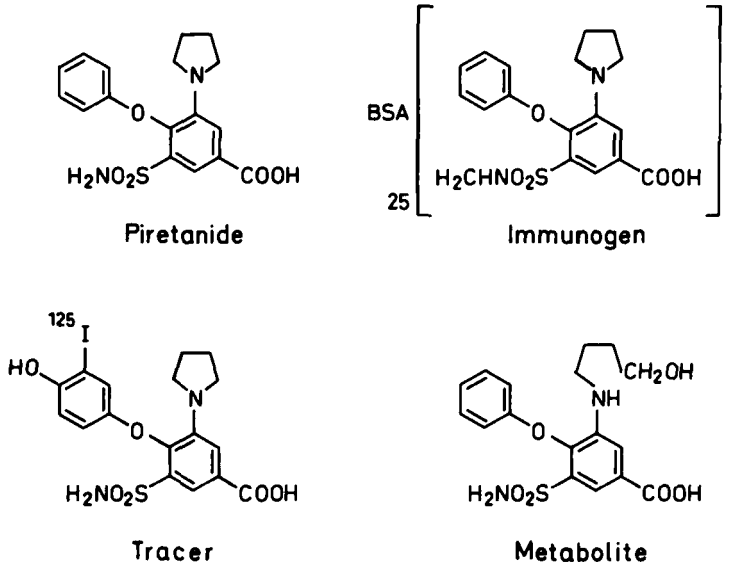

FIGURE 1. Formulas of piretanide, immunogen, tracer and metabolite.

was measured by RIA. The values are given in Tab. 2. The coefficients of variation ranged from 5.0 - 14.1 %.

The interassay imprecision was tested in 3 different control solutions prepared by dissolving weighed amounts in assay buffer. 0.1 ml of these solutions was used for determination of piretanide concentrations in the presence of 0.05 ml human plasma. In ten replicates the interassay imprecision was 3 % at 75 ng/ml, 5.1 % at 35 ng/ml and 6.7 % at 50 ng/ml.

Recovery

The agreement between the mean estimate of a quantity and its true value was determined in a recovery experiment (Tab. 2). The

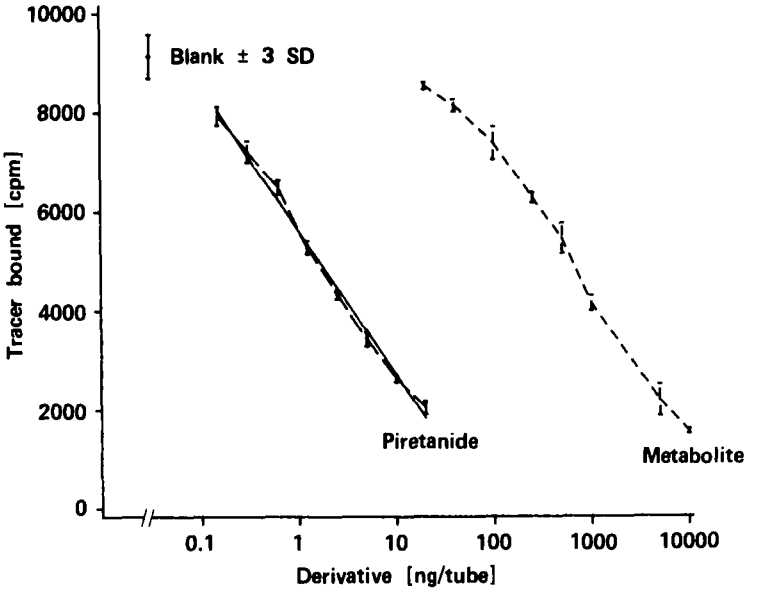

FIGURE 2. Standard curve for piretanide determination and cross-reactivity of the metabolite (for formula see Fig. 1). Assay standard conditions were used. Each point is the mean of 3 observations ± 1 SD. Cross-reactivity of the metabolite is $\leq$ 0.5 %.

relative bias values ranged from -7.7 - 8.4 %. Average recovery was 100.9 %.

One sample t-tests were performed for every concentration listed in Tab. 2. Statistically significant deviations were observed at 20 ng/ml and 200 ng/ml. The reason for these deviations is not clear in retrospect but may be for technical reasons. As both imprecision and bias values are below 10 %, the deviation seems not to be relevant for this method.

Accuracy

The assay accuracy was established by comparing values measured by RIA and HPLC in both human serum and urine. Serum samples

TABLE 2

Recovery of piretanide added to human serum and the values for imprecision and accuracy (bias). Assay standard conditions were used. Concentrations are given in ng/ml. Each value is the mean of 10 replications.

Piretanide added	Mean	Imprecision		Bias
		SD	C.V. %	ng/ml
0	$<$DL	-	-	-
6	6.32	0.89	14.1	+0.32
12	11.64	1.16	9.9	-0.36
20	21.68	1.59	7.3	+1.68
50	51.28	3.04	5.9	+1.28
100	100.6	5.15	5.1	+0.60
200	184.7	9.92	5.4	-15.3
500	519.4	35.26	6.8	+19.4
1000	1012.1	50.35	5.0	+12.1
2000	1916.5	150.7	7.9	-83.5
10000	10164	552.5	5.4	+164

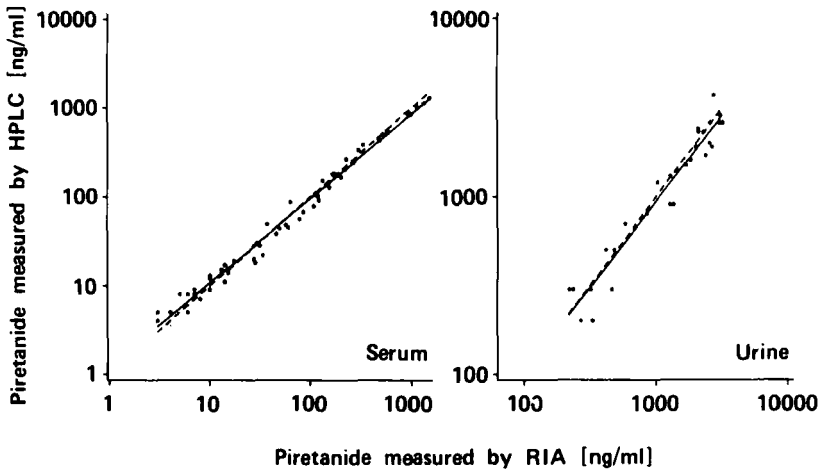

FIGURE 3. Comparison of RIA and HPLC for determination of piretanide. Log-log plot of parallel determinations in serum (n = 57) and urine (n = 30). Four healthy subjects received 6 mg piretanide intravenously. For sample collection see text.

were collected after intravenous injection of piretanide into healthy volunteers at 0.083, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 1.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0 hours. Hourly urine collections were made for 9 hours after drug administration. The scatter plots of method comparisons in serum and urine samples are shown in Fig. 3. The log-log regression calculation resulted in:

$$\ln c_{HPLC} = (0.19 \pm 0.07) + (0.95 \pm 0.015) \ln c_{RIA} \text{ (serum)}$$

$$\ln c_{HPLC} = (0.14 \pm 0.34) + (0.97 \pm 0.05) \ln c_{RIA} \text{ (urine)}$$

In serum both intercept and slope are significantly different from 0 and 1 respectively, leading to 3 % bias at 25 ng/ml and 15 % at 1250 ng/ml.

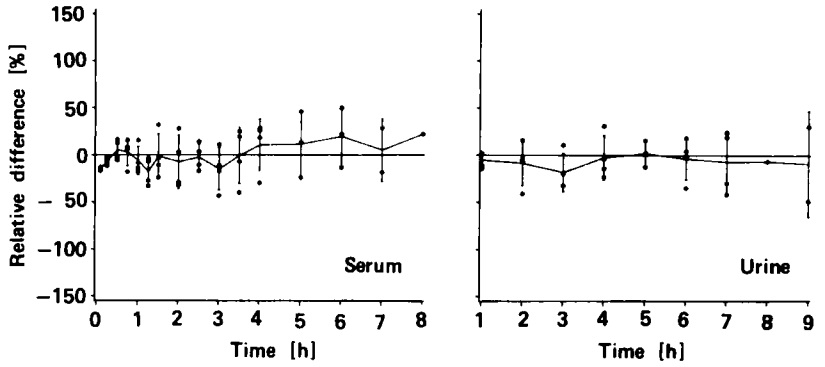

FIGURE 4. Comparison of RIA and HPLC in human serum (n = 57) and urine (n = 30). The relative differences of HPLC and RIA are plotted versus time after medication. The relative difference of the method was calculated as

$$100 \frac{C_{HPLC} - C_{RIA}}{\frac{C_{HPLC} + C_{RIA}}{2}}$$

Positive differences correspond to $C_{HPLC} \geqslant C_{RIA}$

In urine only HPLC values $\geqslant$ 200 ng/ml were used for the regression calculation because the detection limit of the HPLC procedure is 100 ng/ml. There were no significant differences between these two methods.

Time plots of the relative differences (4) of HPLC and RIA are shown in Fig. 4, indicating that there is no time-dependent trend in the estimation of the methods, such as might result from metabolite interference.

DISCUSSION

The immunogen selected yielded a specific antiserum for piretanide determination. So far no interference has been observed in blank samples in healthy subjects and in patients receiving

multiple drug treatment. The method can be used for human serum only because calibration curves in other species are different.

The method has been validated regarding specificity, detection limit and reproducibility. Accuracy was demonstrated by good agreement between HPLC and the RIA determination in human serum and urine. Samples containing concentrations higher than 400 ng/ml have to be diluted. The RIA-method has been used for routine determination of piretanide in samples of clinical trials and in several institutes. It is fast and allows processing of a large number of samples.

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