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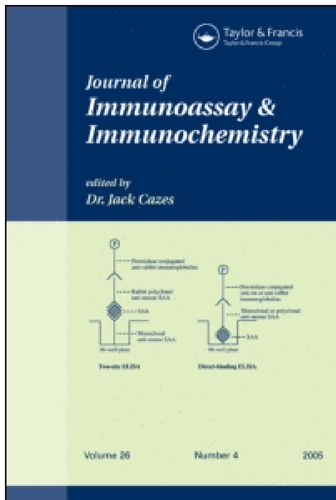

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Direct Micro-Radioimmunoassay of the New Renin Inhibitor Cgp 60536

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DIRECT MICRO-RADIOIMMUNOASSAY OF THE NEW RENIN INHIBITOR CGP 60536

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ABSTRACT

A solid phase method for direct radioimmunoassay in plasma of the new renin inhibitor CGP 60536 has been developed which does not require the extraction of the parent drug with organic solvents. The assay showed a good reproducibility down to plasma concentrations of 0.15 ng/ml (LOQ) with intra- and inter-assay coefficients of variation ≤ 20%. The procedure, which requires only small volumes of plasma (25 μl), is simple to use and well suited for routine analysis. The method allows the investigation of the pharmacokinetics of CGP 60536 in animals and man given low oral doses of the drug. (Key words: CGP 60536; renin inhibitor; microtiter plate; micro-RIA).

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INTRODUCTION

Renin and the angiotensin converting enzyme (ACE) are peptidases of the renin-angiotensin system (RAS). This system is a multi-regulated proteolytic cascade producing potent pressor peptides (1,2). In accordance with the participation of the RAS in the control of blood pressure haemostasis, treatment with ACE inhibitors reduces blood pressure in hypertensive patients (1,3,4). Although ACE inhibitors are generally well tolerated, they give rise to adverse effects in some patients (5). Inhibition of the first enzyme of the RAS, renin, potentially reduces clinical side effects, since renin is highly specific, possessing only one known substrate, namely angiotensinogen (1,2).

CGP 60536 (Figure 1) given as a fumarate salt (CGP 60536B), is a new completely non-peptide low molecular weight renin inhibitor under investigation in the treatment of hypertension and chronic renal failure (6). Animal experiments have clearly shown CGP 60536 to have a high capacity to block renin activity (6). CGP 60536 has been found to be very potent, thereby necessitating a sensitive assay method for the evaluation of its pharmacokinetics. A high-performance liquid chromatography method using fluorescence detection with a limit of quantitation of 4.5 ng/ml in plasma has previously been developed (7) and successfully used for pharmacokinetic investigations in various animal species and in humans. However, due to the high potency of the drug, single oral doses as low as 5 mg are to be administered in humans. This requires a more sensitive analytical method.

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This paper deals with the development of a solid phase micro-radioimmunoassay (micro-RIA) for the direct measurement of CGP 60536 in plasma. Using this method, it has been possible to quantify low concentrations of the drug in man.

MATERIALS AND METHODS

Reagents

CGP 60536B ($C_{30}H_{53}N_3O_6$ + ½·$C_4H_4O_4$, M_r 609.8 as fumarate salt), from Novartis Pharma AG, Basle, Switzerland. CGP 60536B refers to the salt (M_r 609.8) and CGP 60536 refers to the free base ($C_{30}H_{53}N_3O_6$, M_r 551.77). All concentrations are expressed in mass per volume of the free base. ^{125}Iodine-labelled CGP 60536, Bolton-Hunter conjugate (ref. A3-AI-258) (Figure 1), with a specific activity of 2130 Ci/mmol (2 x 60 μCi, lyophilised, reconstituted with 50 μl methanol and 100 μl water), was supplied by ANAWA Laboratories AG, Wangen, Switzerland. Water was purified and deionized using a Milli-Q® Plus Reagent Grade Water Purification System (Millipore, Bedford, MA, USA). All other reagents were of chemical or analytical grade.

Immunogen Preparation

One immunogen was prepared by coupling CGP 60536 to KLH (Keyhole Limpet Hemocyanin) using glutaraldehyde (Figure 1). CGP 60536 and KLH were mixed in borate buffer pH 10 in presence of glutaraldehyde (0.1%) during 2 h at

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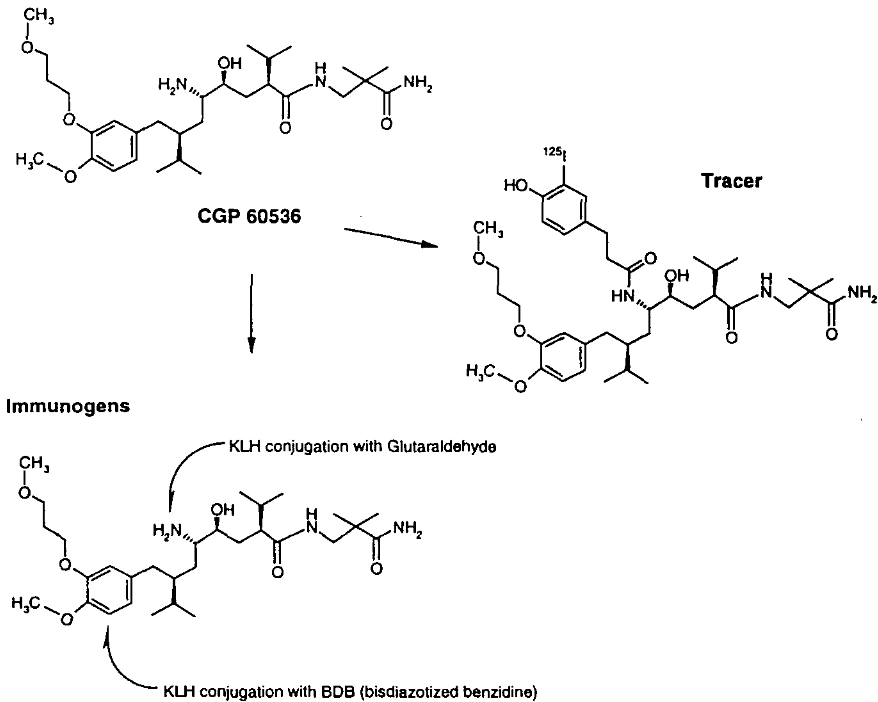

FIGURE 1. Structure of CGP 60536 and synthesis of the immunogens for anti-CGP 60536 antibodies production and of the radio-tracer.

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room temperature allowing reaction between the amino-group of CGP 60536 and one amino-group (ε-lys) of KLH. After addition of a 1 M glycine solution and stirring for 30 min, CGP 60536 conjugated to KLH was separated from free CGP 60536 by centrifugation and chromatography on Sephadex G25 columns. Another immunogen was prepared by coupling CGP 60536 to KLH using bisdiazotized benzidine (BDB). However, this immunogen did not give rise to any proper antibody.

Antisera Production

Antisera against CGP 60536 were raised in New Zealand White rabbits (3 per immunogen) using a standard immunization procedure, namely 300 μg/animal of immunogen in complete Freund's adjuvant at 10 intradermal sites in the back and 200 μg immunogen/animal in incomplete adjuvant for subsequent boosters at 2, 4, 8, 12 and 16 weeks. Blood samples were withdrawn 10 days after each booster in order to assess the production of antibodies. The animals were bled by heart puncture 4 weeks following the last booster (5th month of immunization), and sera (80 to 100 ml from each animal) were stored at -20°C.

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Purification of the polyclonal antibodies

The purification of the anti-CGP 60536 antibodies (from rabbit SB42 at week 20 of immunization) was performed using a standard procedure with ammonium sulphate precipitation. The immunoglobulins were purified on an anion exchanger and by size exclusion chromatography and were then lyophilised. The powder obtained (890 mg) contained 16.3% of proteins which essentially corresponded to IgGs, the purity of which (> 85%) was assessed by electrophoresis (SDS PAGE) and Western-Blot.

Preparation of Standard and Control Plasma Samples

A stock solution of CGP 60536 (50.3 μg/ml) was prepared in water (50 ml) from powder. This solution can be kept at +4°C for at least 7 months without any

alteration. Prior to preparing the standard samples, an aliquot of the stock solution was extemporaneously diluted 1:50 to 1006 ng/ml with drug-free human plasma pool (blank plasma), and then to 62.9 ng/ml by additional sequential ½ dilutions. Starting from this, 12 working standard samples (range 31.4-0.015 ng/ml) were obtained by sequential ½ dilutions in blank plasma.

Control samples for validation of the method were prepared in aliquots of drug-free pooled human plasma (and/or individual plasma) spiked with known amounts of CGP 60536 (range 0.102-314 ng/ml). Pooled and individual plasma were from a local blood bank.

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Radioimmunoassay Procedure

The procedure is based on a competitive micro-radioimmunoassay technique already set up in our laboratory, and which has been successfully used for pharmacokinetic investigations of an antipsychotic drug in humans (8).

Each well of a microtiter plate (flat bottomed 96-well high binding capacity polystyrene microtiter plates, BreakApart module in frame, Nunc, Copenhagen, Denmark) was coated with 100 µl of affinity isolated sheep anti-rabbit IgG (Sigma-Aldrich, ref. R-3661) diluted to 2 µg/ml in 0.1 M sodium carbonate buffer, pH 9.6. The plate was sealed with a plastic film and kept overnight at room temperature. The wells were then emptied and the unoccupied binding sites saturated for 2 h at room temperature with 200 µl of phosphate-buffered saline (PBS) pH 7.3, containing 0.1% Tween 20 (Fluka, ref. 93773) and 1% BSA

(fraction V, Fluka, ref. 05480) (PBSTB). The wells were then automatically washed three times (microplate washer Titertek, Flow Laboratories Ltd, Irvine, Scotland) with PBS containing 0.1% Tween 20 (PBST), and 100 µl of anti-CGP 60536 antibody solution in PBST (1 µg/ml) were added to each well, except three "air blanks". After a 4.5-h incubation at +4°C, the plate was washed three times with PBST. Twenty-five µl/well of standard, control or unknown plasma samples were added in triplicate, immediately followed by 75 µl/well of the tracer ^{125}I-CGP 60536 (6000 cpm/well in PBST). After gentle stirring and an overnight incubation at +4°C, the plate was washed three times with PBST. Subsequently, the plate was fragmented into its individual well components; each well was put into a 5-ml polypropylene tube and then counted (immuno-bound fraction) in a gamma counter (COBRA 5002, Packard, Canberra Industries, Downers Grove, IL, USA).

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All the data processing was performed by means of the computer program RIASMART (Packard). For construction of the standard curve, the RIA results were expressed in terms of %B/Bo (B/Bo values represent the ratio of radiotracer bound to the antibodies in the presence (B) and absence (Bo) of unlabelled CGP 60536), and plotted versus the logarithm of the concentrations of added unlabelled CGP 60536. The curve was drawn by fitting the points by means of a smoothed cubic spline method (RIASMART software from Packard). CGP 60536 concentrations in unknown samples were then automatically calculated from this standard curve.

Assay Performance and Specificity

Assay performance was characterized by assessing precision (intra- and inter-assay variation), accuracy (recovery from spiked samples) and sensitivity (limit of quantitation). For this purpose, aliquots of pooled human plasma were spiked with known concentrations of CGP 60536. One set of samples ranged from 0.102 to 4.9 ng/ml (covering the working range of the calibration curve), and another set of samples ranged from 9.8 to 314 ng/ml (far exceeding the upper part of the calibration curve and thus requiring dilutions prior to the RIA). Each sample was assayed several times, on the same day and/or successive days.

The limit of quantitation (LOQ) of the assay was defined as the lowest concentration that could be determined with a precision of ≤ 20%, and an accuracy within ± 20% of the nominal (given) concentration value (9).

The specificity of the radioimmunoassay was tested by cross-checking with the existing HPLC method (7), using both spiked and actual samples from animal and human studies.

Toxicokinetics in Rats

An oral toxicity study was conducted in rats given oral (gavage) once daily doses of 100, 300 or 1000 mg/kg body weight. Plasma concentrations of CGP 60536 were determined prior to drug administration and at selected time-points until 8 h after dosing.

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Study in Healthy Subjects

Thirty healthy male volunteers (aged 21-48 years) were involved in a single-blind, placebo controlled, randomized, single centre study and were given oral single ascending doses of CGP 60536. Blood samples were withdrawn at selected time-points up to 48 h post-dosing. One blood sample was withdrawn immediately prior to drug administration (time-point 0 h).

The study was conducted at Clin-Pharm Research, Birsfelden, Switzerland, in accordance with the World Medical Association's Declaration of Helsinki, Venice, Hong Kong and Somerset West amendments 1983, 1989 and 1996, and Good Clinical Practice. The study protocol and the subject informed consent forms had been approved by an Ethics Committee. The nature, purpose, and possible risks of the study were fully explained to each subject before obtaining their voluntary written informed consent.

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RESULTS

Precision, Accuracy and Sensitivity of the RIA

Preliminary screening showed that the antiserum from rabbit SB42 (immunized with the glutaraldehyde conjugate) gave the best results as regards antibody titer. This antiserum was therefore selected for purification of the anti-CGP 60536 antibodies. A suitable dilution of antiserum (or purified antibodies) in competitive binding radioassays is that which yields a binding between 30-50% of the total

labelled tracer in the absence of unlabelled material. An optimum dilution of the antibodies purified from serum SB42 was found to be 1 µg/ml. A 6000-cpm concentration per well of tracer was used, allowing for a minimum non-specific binding (less than 1%) and a greater sensitivity of the assay.

Figure 2 depicts a typical standard curve in human plasma covering the concentration range from 0.015 to 31.4 ng/ml.

Table 1 shows the intra- and inter-day precision and accuracy of CGP 60536 measurements in 7 spiked plasma samples in the range 0.102-4.91 ng/ml.

In the within-run assay, coefficients of variation (CVs) for the concentrations from 0.15 to 4.9 ng/ml (working interval) ranged from 3 to 19%. The analytical recoveries (found/given concentrations in %) ranged from 90 to 96%. For the lowest concentration (0.102 ng/ml), the CV was significantly higher (32%) and the recovery showed lower accuracy (118%).

In the between-run assay, the CVs ranged from 10 to 20% and the recoveries from 93 to 101% (concentration range 0.15-4.91 ng/ml).

Accepting both a variability ≤ 20% and recoveries comprised between 80 and 120% (9), 0.15 ng/ml appeared to be the limit of quantitation (LOQ) of the assay. The limit of detection (LOD) of the assay was established to be 0.1 ng/ml (signal different from that observed for a drug-free plasma sample). Samples with concentrations higher than 5 ng/ml have to be diluted prior to the RIA.

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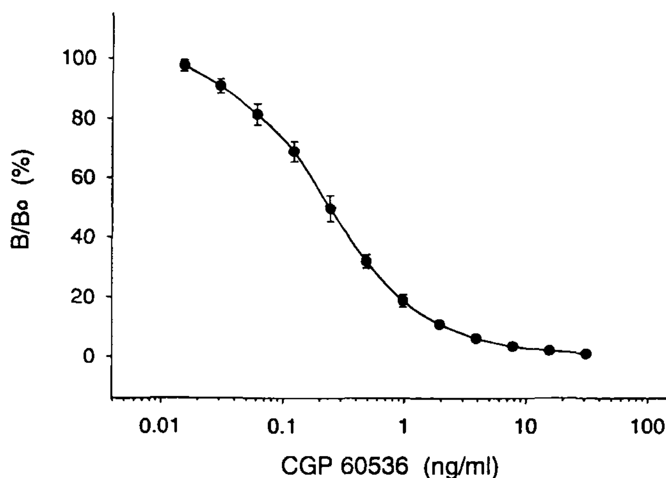

FIGURE 2. Standard curve for CGP 60536 assay in human plasma. Displacement of ^{125}I-CGP 60536 (radiotracer) by increasing amounts of unlabelled drug. B/Bo values represent the ratio of radiotracer bound to the antibodies in the presence (B) and absence (Bo) of unlabelled CGP 60536. Each value is the mean (± SD) of 4 determinations performed on different days.

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Table 2 shows the accuracy and precision of measurements in human plasma samples spiked with high concentrations (range 9.8-314 ng/ml) and which required dilutions before RIA analysis. CVs were in the range 9-20% and recoveries in the range 91-110%, demonstrating that measurement of high concentrations (following large sample dilutions) was also performed with good accuracy and precision.

Matrix Effect

Inter-species variabilities (i.e. matrix effects) were investigated using rat and dog plasma as these species were used in toxicokinetic studies. Standard curves

TABLE 1.

PRECISION AND ACCURACY OF THE ASSAY IN HUMAN PLASMA. Human plasma samples spiked with known amounts of CGP 60536 were assayed 5-6 times in triplicates on the same day. For inter-assay variation, each sample was measured in triplicates on different days. (Recovery = found/given x 100).

Intra-assay measurements

Given (ng/ml)	Found ± SD (ng/ml)	CV (%)	Recovery (%)	n
0.102	0.121 ± 0.039	32	118	5
0.153	0.138 ± 0.026	19	90	6
0.307	0.289 ± 0.035	12	94	6
0.614	0.586 ± 0.020	3	95	6
1.23	1.16 ± 0.11	9	94	6
2.46	2.37 ± 0.28	12	96	6
4.91	4.66 ± 0.56	12	95	6

Inter-assay measurements

Given (ng/ml)	Found ± SD (ng/ml)	CV (%)	Recovery (%)	n
0.102	0.111 ± 0.042	38	109	33
0.153	0.147 ± 0.028	19	96	27
0.307	0.294 ± 0.059	20	96	30
0.614	0.619 ± 0.115	19	101	27
1.23	1.22 ± 0.15	12	99	35
2.46	2.29 ± 0.34	15	93	25
4.91	4.55 ± 0.45	10	93	14

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TABLE 2.

PRECISION AND ACCURACY OF THE ASSAY IN HUMAN PLASMA REQUIRING DILUTIONS. Human plasma samples spiked with high amounts of CGP 60536 were assayed on several occasions (n), each time in triplicates. (Recovery = found/given x 100).

Given (ng/ml)	Found ± SD (ng/ml)	CV (%)	Recovery (%)	n
9.82	10.8 ± 1.25	11	110	6
19.6	21.3 ± 2.44	11	109	8
39.3	41.2 ± 3.8	9	105	8
78.6	76.6 ± 15.3	20	97	7
157	165 ± 14	9	105	9
314	287	-	91	2

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prepared in rat or dog plasma were comparable to those in human plasma showing similar Bo values. Precision, accuracy and LOQ (Table 3) were comparable to those observed in human plasma.

Specificity

The specificity of the RIA method was assessed by cross-comparing concentration values obtained using this RIA and the previously developed HPLC method (7). This cross-comparison was performed on both spiked plasma samples and actual plasma samples from toxicokinetic (rat) and clinical (human) studies. Though no inter-species matrix effects were observed, standard curves and quality control samples were prepared in the appropriate species plasma (i.e. rat or human) and processed in the same way for HPLC measurements.

TABLE 3.

PRECISION AND ACCURACY OF THE ASSAY IN RAT PLASMA. Rat plasma samples spiked with known amounts of CGP 60536 were assayed on several days (n), each time in triplicates. (Recovery = found/given x 100).

Given (ng/ml)	Found ± SD (ng/ml)	CV (%)	Recovery (%)	n
0.153	0.160 ± 0.048	30	105	3
0.307	0.297 ± 0.034	11	97	3
0.614	0.597	-	97	2
1.23	1.10 ± 0.12	11	98	3
2.46	2.65	-	108	2
4.91	4.37 ± 0.67	15	89	3

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The RIA and HPLC values measured in human plasma samples spiked with known amounts of CGP 60536 (range 4.9-314 ng/ml) were in excellent agreement as demonstrated by the equation of the least-squares linear regression line, RIA = 1.0·HPLC + 3, r = 0.993 (n = 20).

As displayed in Figure 3, there was also a good agreement between the HPLC and RIA plasma concentration values measured in rats given oral doses of the drug (RIA = 0.97·HPLC - 6.5, r = 0.964).

A comparison between the two methods was also performed using human plasma samples from healthy volunteers given an oral dose of the drug. Figure 4 shows one plasma profile measured by both RIA and HPLC methods. The two profiles were nearly identical, demonstrating the good agreement between the two

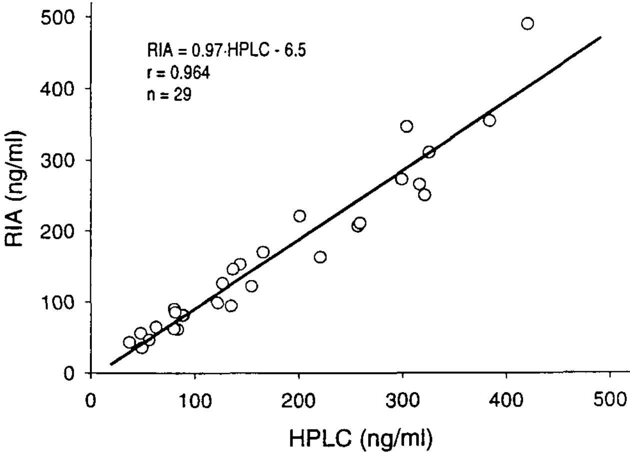

FIGURE 3. Cross-comparison between concentrations measured by RIA and HPLC in rats given oral doses of CGP 60536B.

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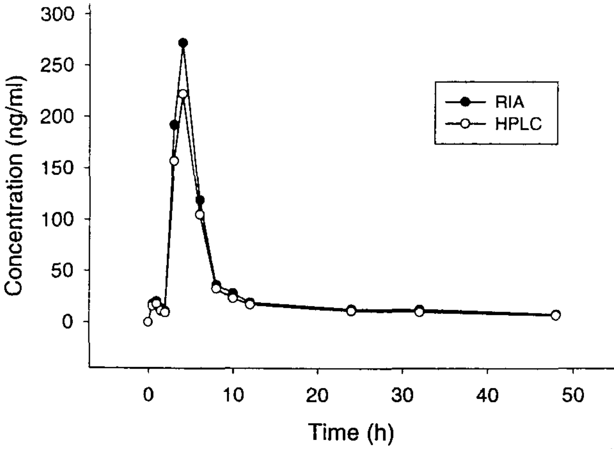

FIGURE 4. Example of plasma concentrations versus time profile measured by RIA and HPLC in one healthy subject given an oral dose of 80 mg CGP 60536B.

methods. Some of the concentrations were below the LOQ of the HPLC method (i.e. 4.5 ng/ml). However, though these values might have been determined with lower accuracy by HPLC, the correlation was still excellent (linear regression RIA = 1.06·HPLC - 0.06, r = 0.930). No drug was detected in any of the plasma samples taken just prior to drug administration (time-point 0 h), confirming the absence of any individual matrix effect. The plasma concentrations increased rapidly, reaching peaks at around 1-3 h post-administration. CGP 60536 was still detected in plasma up to 48 h post-dosing.

It happened that RIA values were found to be slightly higher than HPLC findings, but these discrepancies were considered to have no relevant impact on the subsequent pharmacokinetic evaluations (to be published separately).

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DISCUSSION

Simple and rapid in use, this direct competitive micro-radioimmunoassay provides numerous practical advantages, rendering it suitable for routine applications. The use of microtiter plates and the ability to measure the renin inhibitor CGP 60536 in plasma samples without prior extraction with organic solvents facilitates a rapid sample turnover. Accordingly, one technician can easily handle approximately 100 samples per run.

The delicate step of separation of free from bound fraction usually met with classical liquid phase radioimmunoassays was eliminated. It was well established that, of the available separation procedures, the best in terms of efficiency (low

assay blank and completeness of separation of bound fraction) were solid phase systems (10). The proposed RIA procedure obviates the drawbacks met with all other separation techniques (i.e. uncomplete separation, poor reproducibility, non-comparability of concentrations of the measured analyte (10-13)).

The last operation of the assay, consisting of fragmenting the microplate into individual wells and placing each well into a tube for radioactivity counting, is easily performed. However, this step could be eliminated using recently marketed microtiter plate counters, which proved to be convenient and reliable. A non-radioactive procedure, using an enzyme-linked tracer (CGP 60536 labelled with horseradish peroxydase) was also initiated. However, this assay has not been further developed due to reliability/reproducibility problems and lack of sensitivity. Even if enzyme immunoassay technology is often preferred to isotopic labels (the principle reason being the elimination of radiation hazard of isotopes) our radioimmunoassay proved to be easy to develop, very convenient to use and more efficient and reliable than the procedure using the enzyme technology.

Owing to their greater specificity, monoclonal antibodies were generally preferred to polyclonal antibodies (11) and double antibody sandwich assays to competitive assays (14). Nevertheless, our competitive RIA demonstrates that polyclonal antibodies still represent a useful and valid alternative.

The method was found to be also valid for measurements in rat or dog plasma so it can be used to investigate pharmacokinetics of CGP 60536 in these species. Though no inter-species matrix effect was observed, it is however recommended,

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when available, to prepare the standard samples (as well as the quality control samples) in plasma coming from the same species as the unknown samples to be analysed.

The application of the method to the measurement of actual clinical samples indicated that the limit of quantitation of the assay, which is 30-fold more sensitive than the HPLC method previously developed (7), is now sufficient to perform pharmacokinetic evaluations even at low doses of the drug. The slight occasional overestimations (in comparison to HPLC findings) of some plasma levels could be due to freezing and thawing cycles of the plasma samples. The fact that these actual samples were measured first by HPLC and only later on by RIA, and that in consequence underwent several freeze-thaw cycles might explain the discrepancies. Indeed, the storage (freezing) conditions of the plasma samples were shown to potentially seriously impact accuracy of CGP 60536 determination (7). It was also assumed that there was no metabolite cross-reactivity with the antibody because the HPLC and RIA measurements were nearly identical.

A method coupling the present RIA to the HPLC (7) was set up and validated in our laboratory so as to investigate potential causes for discrepancies and to further ascertain the specificity of the RIA. This technique which combined the high sensitivity of the present RIA with the specificity of the HPLC, overall, gave comparable results to those obtained using the direct RIA alone. This indicates that the cause for discrepancies was more likely due to sample storage conditions than to the RIA itself.

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It can be concluded that the present radioimmunoassay is specific, sensitive and ideally suitable for the determination of CGP 60536 in animal toxicokinetic studies as well as in human clinical studies.

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