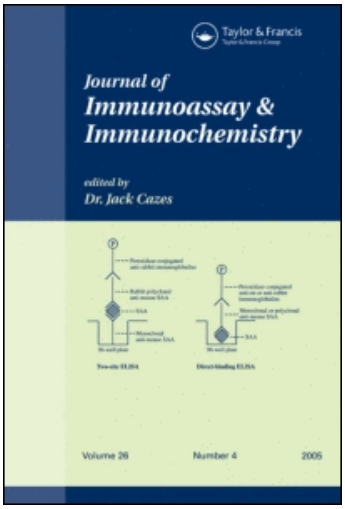

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Lefèvre, Gilbert , Duval, Martine , Botta, Luigi and Godbillon, Jacques(1996) 'Direct Microtitre Plate Radioimmunoassay of Savoxepine in Unextracted Plasma', Journal of Immunoassay and Immunochemistry, 17: 1, 29 — 46
To link to this Article: DOI: 10.1080/01971529608005777
URL: http://dx.doi.org/10.1080/01971529608005777

JOURNAL OF IMMUNOASSAY, 17(1), 29–46 (1996)

DIRECT MICROTITRE PLATE RADIOIMMUNOASSAY OF SAVOXEPINE IN UNEXTRACTED PLASMA

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ABSTRACT

An original solid phase method for direct radioimmunoassay of the antipsychotic savoxepine (CGP 19 486 A) in plasma has been developed which does not require the extraction of the parent drug with organic solvents. The assay showed good reproducibility over the working concentration range 1.9-30.6 nmol/l with intra- and inter-assay coefficients of variation ≤ 16 %. The procedure, which requires only small volumes of plasma (10 µl), is simple to handle and well suited for routine analysis. The method allowed to investigate the pharmacokinetics of savoxepine in schizophrenic patients given low oral doses of the drug. (Key words : CGP 19 486 A ; savoxepine ; neuroleptic ; microtiter plate ; micro-RIA).

INTRODUCTION

Central dopamine receptor blockers are clinically efficacious antipsychotics, but most of them elicit extrapyramidal side effects (EPS) like Parkinson syndrome, tremor, stiffness or akathisia. Savoxepine, the methanesulfonate salt of a

tetracyclic cyano-dibenzoxepino-azepine derivative, is a novel dopamine antagonist.

Animal experiments have clearly shown savoxepine to have a high capacity to block the brain dopamine (DA) D_2-receptors (1,2). This characteristic has been confirmed indirectly in man by the observation of a strong antipsychotic effect in acute episodes of schizophrenia, and by an increase in serum prolactin levels (3). Savoxepine principal interest lies in the potent and highly preferential blockade of DA D_2-receptors in the hippocampus, believed to be related to the antipsychotic activity (4,5). This affinity for hippocampal DA D_2-receptors is, on the average, 17 times greater than the one for the striatum receptors (6), the blockade of which results in EPS. In consequence, savoxepine is expected to be better tolerated than the classical neuroleptics even though Moller et al. (7) and Wetzel et al. (8), reported that savoxepine might sometimes bring about untoward EPS in patients.

Due to the high potency of savoxepine, low maintenance doses (0.5 mg/day or less) are needed in patients with schizophrenia. Thus, the assessment of pharmacokinetics of this compound requires a sensitive analytical method.

This paper deals with the development of an original solid phase micro-radioimmunoassay (micro-RIA) for the direct measurement of savoxepine in body fluids. Using this method, it has been possible to quantify low concentrations of the drug in plasma.

MATERIALS AND METHODS

Supplies and chemicals

Flat bottomed 96-well high binding capacity polystyrene microtitre plates (BreakApart module in frame) were from Nunc, Copenhagen, Denmark. The

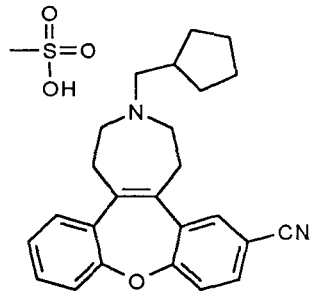
automatic Titertek microtitre plate washer was from Flow Laboratories Limited, Irvine, Scotland. The single detector gamma counting system COBRA 5002 controlled by an IBM computer was from Packard, Canberra Industries, Downers Grove, IL, USA. The software (RIASMART) for automated standard curve construction and calculations of the concentrations in the unknown samples was also from Packard.

Savoxepine (CGP 19 486 A, $C_{25}H_{26}N_2O.CH_4O_3S$, molecular weight 466.6) and the five potential metabolites of the drug, CGP 45 628 A ("hydroxy", $C_{25}H_{26}N_2O_2.HCl$, molecular weight 422.9), CGP 45 629 ("N-oxide", $C_{25}H_{26}N_2O_2$, molecular weight 386.5), CGP 21 744 A ("N-dealkyl", $C_{19}H_{16}N_2O.HCl$, molecular weight 324.8), CGP 45 502 A and CGP 45 503 A ("cis-diol" and "trans-diol", $C_{25}H_{26}N_2O_3.HCl$, molecular weight 438.9) (Figure 1), were from Ciba-Geigy Limited, Basle, Switzerland. ^{125}Iodine-labelled savoxepine-tyrosine methyl ester conjugate (Figure 2), with a specific activity of 2200 Ci/mmol (24 μCi/ml, 5 ml), was supplied by ANAWA Laboratories AG, Wangen, Switzerland. Affinity purified anti-guinea pig IgG raised in rabbit, were purchased from SIGMA (ref. G 4143), bovine serum albumin (for RIA) was from Behring (ref. ORHD/2021) and polyoxyethylene sorbitanmonooleate (Tween 80) was from Fluka (ref. 93780). All other reagents were of chemical or analytical grade.

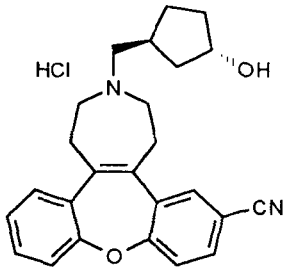
Immunogen Preparation

CGP 29 313 (Figure 2), the carboxylic acid derivative of CGP 19 486, was coupled to bovine serum albumin (BSA) by the mixed anhydride procedure (9).

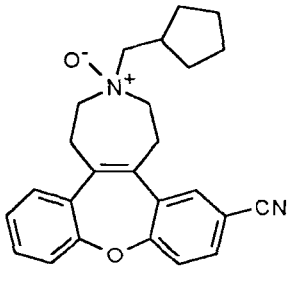
CGP 29 313 was obtained from savoxepine by saponification in 2N NaOH. The carboxylic acid derivative (146 mg, 0.3 mmol) was suspended in 3 ml of dioxane. After addition of 135 mg (0.3 mmol) of tri-n-butylamine and 45 mg (0.3 mmol) of



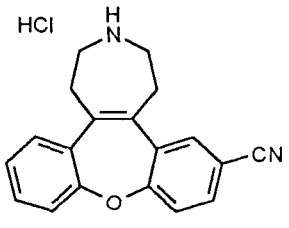
CGP 19 486 A
Savoxepine methanesulfonate



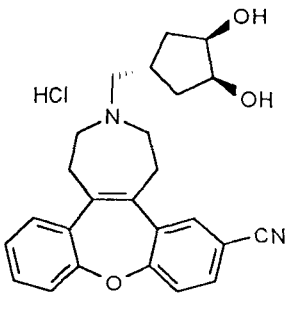
CGP 45 628 A



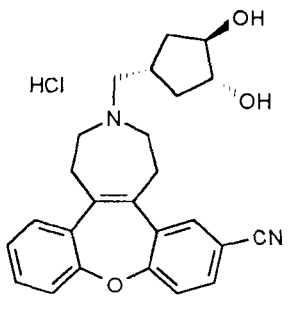
CGP 45 629



CGP 21 744 A



CGP 45 502 A



CGP 45 503 A

FIGURE 1. Structure of savoxepine methanesulfonate (CGP 19 486 A) and of its five potential metabolites.

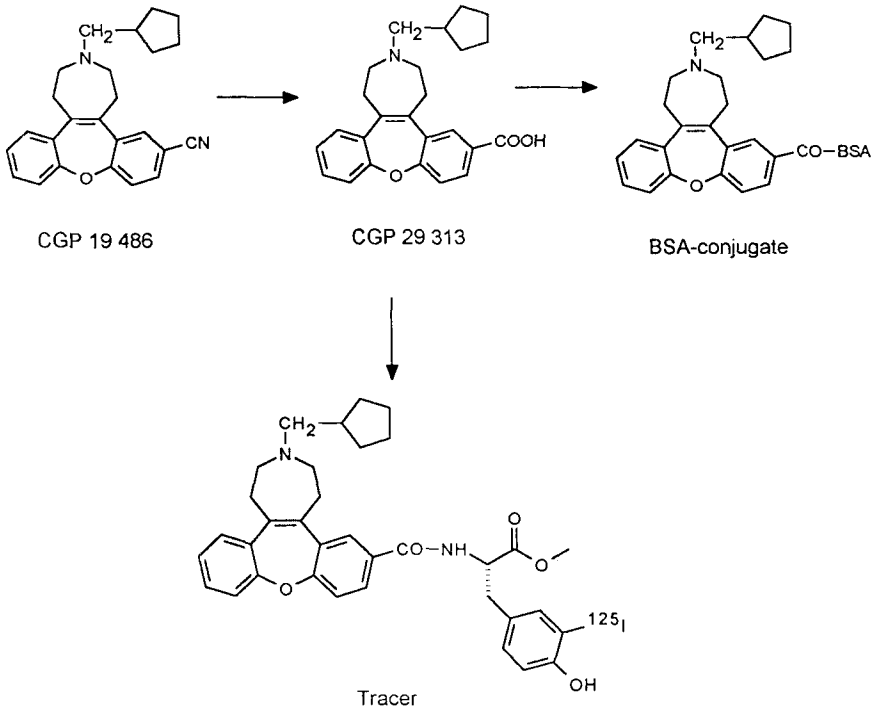


FIGURE 2. Synthesis of the immunogen for anti-savoxepine antisera production and of the tracer.

isobutyl chlorocarbonate, the substance rapidly went into solution. The reaction mixture was stirred at room temperature for a total of 4 min and then rapidly added to a well stirred, ice-cooled solution of 420 mg BSA (Behringer Werke AG) in 26 ml water/dioxane (1/1) and 0.4 ml 1N NaOH. Stirring and cooling were continued for a total of 4 h. After 1 h, 0.2 ml of 1N NaOH was added. The reaction mixture was transferred into a spectrapore membrane tube (No. 4, Labomatic AG, Allschwil, Switzerland) and dialysed for 24 h against deionized water. Thereafter, the solution was lyophilized, yielding 450 mg of conjugate (Figure 2).

The number of CGP 29 313-residues covalently bound to BSA was determined by colorimetry using trinitrobenzenesulfonic acid (10) or dinitrofluorobenzene (11) as the reagents. The conjugate was found to contain about 30 haptens per molecule of BSA.

Antisera Production

Antisera against savoxepine were raised in guinea pigs (Dunkin-Hartley, SPF-bred) using Freund's adjuvant. For primary immunization, 50 µg of immunogen per animal were injected intradermally at about 5 sites in the back. The emulsions with adjuvant were prepared as recommended by the supplier (Freund's adjuvant : immunogen dissolved in 0.5 ml NaCl 0.9 % + 1 ml of complete Freund's adjuvant). Boosters were given monthly (25 µg immunogen/animal) alternating incomplete Freund's adjuvant (i.d.) with physiological saline (s.c., 1 ml). After 6 months, one week following the last booster, the animals were bled by heart puncture under ether anaesthesia. Between 5 and 15 ml of serum were obtained from each animal. Sera were stored at -20°C.

Standard and Control Plasma Samples Preparation

Savoxepine standard stock solution (440 µmol/l) was prepared in ethanol (20 ml) from powder. The solution can be kept at +4°C for at least 5 months without any alteration. Prior to preparing standards, an aliquot of the stock solution was extemporaneously diluted to 428.6 nmol/l with drug-free human plasma pool (blank plasma). Starting from this, twelve working standards (range 0.105-214.3 nmol/l) were obtained by sequential ½ dilutions in blank plasma.

Control samples were prepared in drug-free human pooled and individual plasma spiked with known amounts of CGP 19 486 A (range 0.96-61.2 nmol/l). Pooled and individual plasma were from a local blood bank.

Radioimmunoassay Procedure

Each well of the microtitre plate was coated with 100 µl of rabbit anti-guinea pig IgG diluted to 2 µg/ml in 0.1 M sodium carbonate buffer, pH 9.6. The plate was kept overnight at room temperature, sealed with a plastic film. The wells were then emptied and the unoccupied binding sites saturated for 1 h at room temperature with 250 µl of phosphate-buffered saline (PBS) pH 7.3, containing 0.1 % Tween 80 and 1 % BSA (PBSTB). The wells were then automatically washed three times (microplate washer Titertek) with PBS containing 0.1 % Tween 80 (PBST) and 100 µl of anti-savoxepine antiserum diluted 1/30000 in PBSTB were added to each well, except three "air blanks". After a 5-h incubation at +4°C, the plate was washed three times with PBST.

Ten µl of standard, control or unknown plasma samples were added in triplicate, immediately followed by 100 µl of ^{125}I-savoxepine (2000 cpm/well in PBSTB). After 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.

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 (corrected for non-specific binding), and plotted versus the log of the concentrations of added unlabelled savoxepine. The curve was drawn by fitting the points with a four-parameter logistic method. Savoxepine 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, pooled human plasma samples were spiked with 0.96, 1.27, 1.91, 3.83, 7.65, 15.3, 30.6 and 61.2 nmol/l (given concentrations). Each sample was assayed several times, on the same day and on successive days.

The specificity of the anti-savoxepine antiserum was tested with the five potential metabolites of CGP 19 486 A (Figure 1). The cross-reactivity was estimated according to the method of Abraham (12), as the ratio of the IC_{50} values (inhibiting concentrations of savoxepine or potential cross-reactants which decrease the binding of labelled savoxepine by 50 %).

Subjects

Ten patients (aged 25-47 years) and six patients (aged 21-42 years) with a diagnosis of chronic schizophrenia were given oral 1 and 1.5 mg single doses of savoxepine methanesulfonate, respectively. Blood samples were withdrawn at selected time-points up to 72 h post-dosing. One blood sample was withdrawn immediately prior to administration (time-point 0 h).

The study protocols were approved by the Ethical Committees of the Centre Hospitalo-Universitaire de Montpellier, France, and of the Hospital San Raffaele, Milan, Italy. The nature, purpose, and possible risks of the study were fully explained to each patient (or the legally responsible person) before obtaining their voluntary written informed consent.

RESULTS

Precision, Accuracy and Sensitivity of the RIA

Preliminary screening showed that the antiserum from guinea pig No 15 gave the best results as regards antibody titer. A suitable dilution of antiserum 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 antiserum 15 was found to be 1/30000. A 2000-cpm concentration per well of tracer was used, allowing for a minimum non-specific binding (less than 5 %) and a greater sensitivity of the assay.

Figure 3 depicts a typical standard curve in human plasma covering the range from 0.105 to 214.3 nmol/l.

Table 1 shows the precision and accuracy of savoxepine measurements in 8 spiked plasma samples.

In the within-run assay, coefficients of variation (CVs) for the concentrations from 1.91 to 30.6 nmol/l (working range) ranged from 9.4 to 14.1 %. The analytical recoveries (found/given concentrations in %) ranged from 96 to 102 %. For the two lowest concentrations (0.96 and 1.27 nmol/l), the CVs were significantly higher (30.3 and 22.6 %) and the recoveries showed lower accuracy (116 and 121 %). Accepting both a variability $\leq$ 15 % and recoveries comprised between 80 and 120 %, 1.9 nmol/l appeared to be the limit of quantitation (LOQ) of the assay. The limit of detection (LOD) of the assay was established to be 0.9 nmol/l (signal different from that observed for a drug-free plasma sample). Samples with concentrations higher than 30 nmol/l have to be diluted with blank plasma prior to the RIA.

Only concentrations which were accurately and precisely measured in the within-run assay were considered for inter-assay variability assessment. In the between-run assay the CVs ranged from 10.7 to 16.0 % and the recoveries from 99 to 107 % (concentration range 1.91-30.6 nmol/).

Specificity

Figure 4 depicts the displacement curves obtained with the five potential metabolites of savoxepine compared to that obtained with CGP 19 486 A. Only

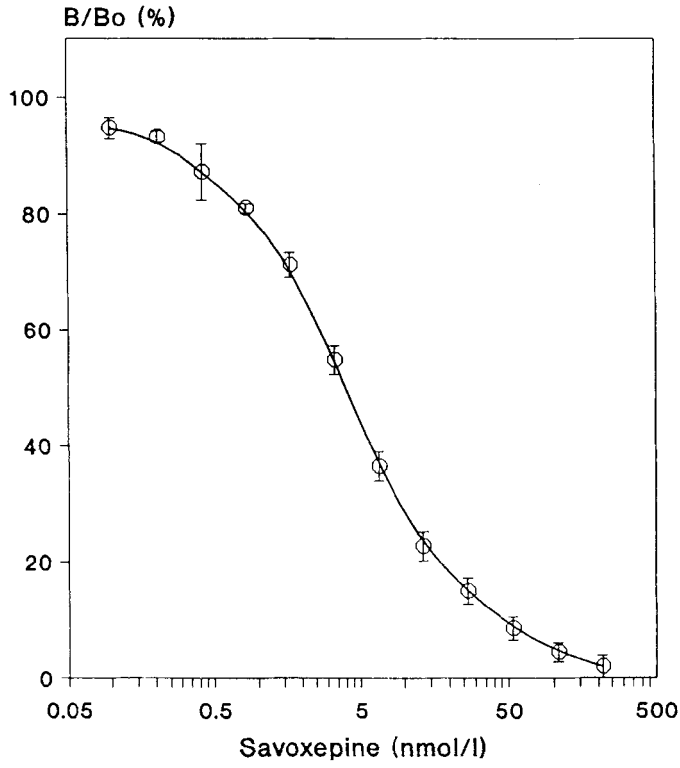


FIGURE 3. Standard curve for savoxepine assay in human plasma. Displacement of ^{125}I-savoxepine from antiserum by increasing amounts of unlabelled savoxepine. B/Bo values represent the ratio of radiotracer bound to the antibodies in the presence (B) and absence (Bo) of unlabelled savoxepine. Each value is the mean (± SD) of 3 determinations performed on different days.

CGP 45 628 A cross-reacted but to a small extent. The percentage of cross-reaction was only 0.8 %.

Matrix Effect

Inter-subject and inter-species variabilities (i.e. matrix effects) were investigated using human and rat plasma.

TABLE 1.

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

Intra-assay measurements

Given (nmol/l)	Found ± SD (nmol/l)	CV (%)	Recovery (%)	n
0.96	1.11 ± 0.34	30.3	116	6
1.27	1.54 ± 0.35	22.6	121	6
1.91	1.95 ± 0.26	13.1	102	6
3.83	3.77 ± 0.36	9.5	98	6
7.65	7.67 ± 0.72	9.4	100	6
15.3	14.7 ± 1.6	10.8	96	6
30.6	30.6 ± 4.3	14.1	100	6
61.2	57.2 ± 17.6	30.7	94	6

Inter-assay measurements

Given (nmol/l)	Found ± SD (nmol/l)	CV (%)	Recovery (%)	n
1.91	1.93 ± 0.27	14.1	101	6
3.83	3.83 ± 0.42	11.0	100	6
7.65	7.62 ± 0.81	10.7	99	6
15.3	16.4 ± 1.8	11.1	107	6
30.6	32.4 ± 5.2	16.0	106	5

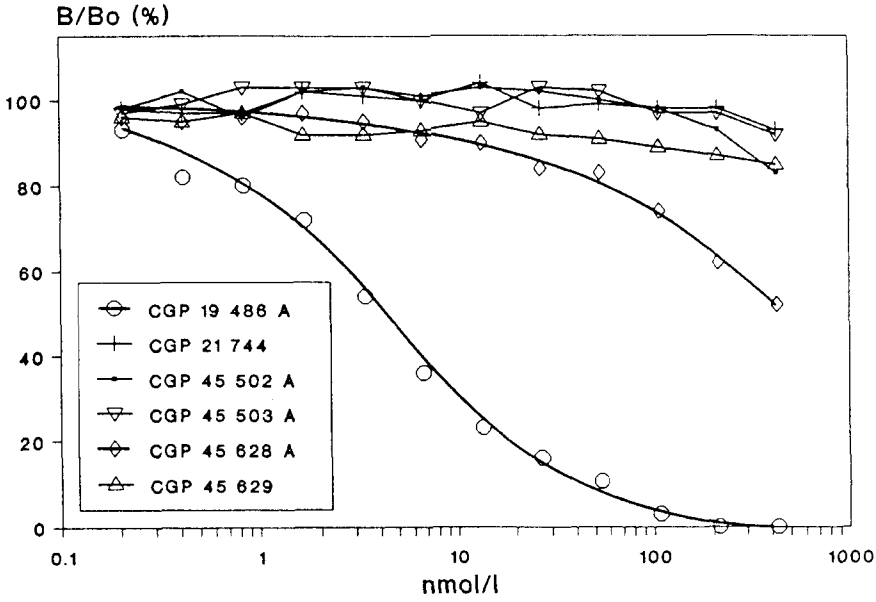


FIGURE 4. Study of possible cross-reactions of potential metabolites with anti-savoxepine antibodies. Displacement curves obtained with increasing amounts of the five compounds compared to that obtained with savoxepine. B/Bo values represent the ratio of radiotracer bound to the antibodies in the presence (B) and absence (Bo) of inhibitor.

Six individual drug-free human plasma samples were spiked with various amounts of CGP 19 486 A in the range 3.8-30.6 nmol/l. There was no inter-subject variability due to individual matrix effects, the CVs being similar to those obtained with pooled plasma.

Since the rat is a species often used in toxicokinetic studies, standard curves were also constructed in rat plasma. They differed from those obtained in human plasma by an enhanced binding of savoxepine (the Bo value was approximately

TABLE 2.

PRECISION AND ACCURACY OF THE ASSAY IN RAT PLASMA. Seven rat plasma samples spiked with known amounts of savoxepine were assayed 6 to 11 times in triplicates (intra-assay values).

Given (nmol/l)	Found ± SD (nmol/l)	CV (%)	Recovery (%)	n
0.96	1.32 ± 0.31	23.9	138	6
1.91	2.25 ± 0.44	19.9	117	11
3.83	4.08 ± 0.70	17.3	106	9
7.65	7.93 ± 1.15	14.5	103	10
15.3	15.8 ± 1.91	12.1	103	9
30.6	29.7 ± 2.73	9.2	97	11
61.2	56.5 ± 5.23	9.2	92	10

twice higher). However, precision, accuracy and LOQ (Table 2) were comparable to those obtained in human plasma.

Application of the Method to Pharmacokinetic Investigations

Plasma samples from schizophrenic patients given oral doses of 1.0 (n = 10) or 1.5 mg (n = 6) savoxepine were analysed by this RIA method. The mean plasma concentrations are displayed in Figure 5. No savoxepine was detected in plasma samples taken just prior to drug administration (time-point 0 h), confirming the specificity of the assay and the absence of any individual matrix effect. Then, the concentrations increased rapidly in the two groups of patients, reached a peak (3.9 and 4.9 nmol/l, respectively) and then decreased sharply in a first phase and more slowly afterwards.

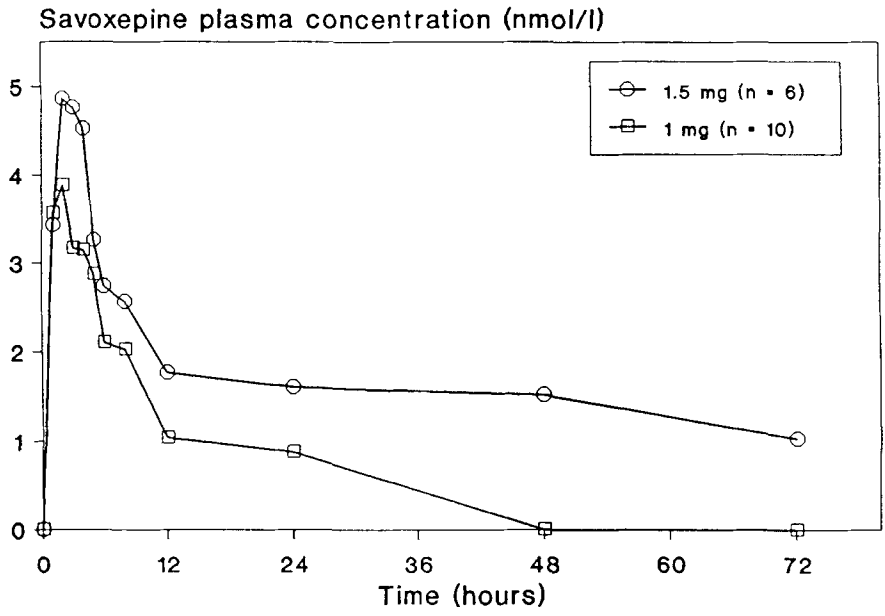


FIGURE 5. Mean savoxepine plasma concentrations in 10 and 6 patients given single oral doses of 1.0 or 1.5 mg savoxepine methanesulfonate, respectively.

DISCUSSION

Simple and rapid to handle, this original direct competitive micro-radioimmunoassay provides numerous practical advantages, rendering it suitable for routine applications. The use of microtitre plates and the ability to measure savoxepine in plasma samples without prior extraction with organic solvents facilitates a rapid sample turnover. Accordingly, approximately 150 samples could be easily handled by one technician per run.

The delicate step of separation of free from bound fraction usually met with classical radioimmunoassays, is eliminated. It is now well established that, of the

available separation procedures, the best in terms of efficiency (low assay blank and completeness of separation of bound fraction) are solid phase systems (13). 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 (13-16)).

The last operation of the assay, consisting in 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.

Considering the high specificity of the anti-savoxepine polyclonal antibodies, the assay allows direct measurement of the drug in plasma without any prior extraction and purification of the analyte, despite the presence of the N-oxide metabolite (CGP 45 629). For the same reason, the method could be used in urine to determine the excretion of the parent compound. This would only require a few minor modifications of the procedure. The hydroxy metabolite (CGP 45 628 A) which is present in urine, only slightly cross-reacted in the assay (0.8 %) and is therefore not expected to interfer with the measurement of urinary savoxepine. Owing to their greater specificity, monoclonal antibodies are generally preferred to polyclonal antibodies (14) and double antibody sandwich assays to competitive assays (17). 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 plasma so it can be used to investigate pharmacokinetics of savoxepine in this species. However, despite the relatively low volume of plasma sample used in the assay (1 volume versus 10 volumes of buffer), an inter-species matrix effect was observed. The

standard samples must thus be prepared 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 indicates that the limit of quantitation of the assay was sufficient to perform pharmacokinetic evaluations even at low doses of drug. Though some data points were close to or below the quantitation limit of the method, it could be shown that clearance of savoxepine was low, which is in agreement with the time-effect courses previously described (6). Data on the elimination half-life of savoxepine in human have never yet been obtained, because plasma levels could not be measured by the available techniques. The present RIA will now allow these determinations. It may be expected that the sensitivity of the method will be increased in the future, allowing determination of plasma levels much lower than the present LOQ.

A non radioactive procedure, using an enzyme-linked tracer (savoxepine labelled with horseradish peroxydase) was firstly chosen. However, this assay was about 100-fold less sensitive than the present RIA.

To our knowlegde, no such RIA design on microtiter plates, used in routine for clinical study purposes, has so far been reported.

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