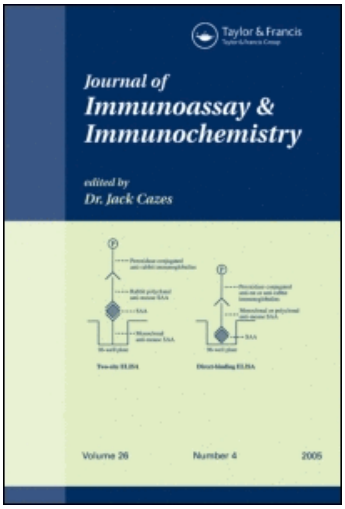

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RADIOIMMUNOASSAY FOR THE SYNTHETIC ERGOLINE DERIVATIVE CABERGOLINE IN BIOLOGICAL FLUIDS

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ABSTRACT

An antiserum against cabergoline, a powerful dopamine-agonist under clinical trials for the treatment of Parkinson's disease and hyperprolactinemia, has been raised in rabbits by immunization with an immunogen produced by conjugation of cabergoline to bovine serum albumin.

The antiserum was able to bind a derivative of cabergoline labelled with tritium and was able to distinguish the drug molecule from some of its close related compounds and from other agents that could be simultaneously present in plasma from patients undergoing treatment with cabergoline.

The antiserum and the tritium labelled hapten were used to develop a radioimmunoassay for cabergoline determination in human plasma and urine.

A linear relationship between cabergoline added and % radioactivity bound was found in the range 1.9-500 pg/tube.

The addition in the assay of 200 μl human plasma or 25 μl urine did not affect the specific and the non-specific binding of the radiolabelled hapten so enabling us to obtain a final sensitivity of about 12 pg/ml plasma and 120 pg/ml urine. The assay was validated in terms of reproducibility, precision and accuracy over the whole range of concentrations tested both in plasma and urine.

The plasma concentrations at the steady state in a patient with Parkinson's disease who had received the drug at single oral daily doses of 3, 5 and 7 mg were determined using the assay.

(KEY WORDS: Cabergoline, Analysis in biological fluids, Radioimmunoassay, Dopamine-agonists.)

INTRODUCTION

Cabergoline (FCE 21336), N-[3-(dimethylamino)propyl]-N-(ethylamino)carbonyl-6-(2-propenyl)-ergoline-8ß-carboxamide is a recently developed synthetic ergoline derivative with dopamine-agonist activity (1), which has been shown to induce long-lasting inhibition of prolactin secretion in hyperprolactinemic patients (2). A single oral dose of 600 µg of cabergoline is effective in decreasing the plasma levels of prolactin for up to seven days (3). The compound has also entered clinical trials for the treatment of Parkinson's disease where, however, higher doses are used (4). Preliminary studies carried out in healthy volunteers with labelled cabergoline had shown, within 24 h after administration of a single dose of 0.6 mg, plasma levels of radioactivity in the range 80-800 pg eq/ml, excretion of the radioactivity mainly by fecal route (about 76% of the dose) and biotransformation of the drug into several metabolites in urine (7).

These findings made it evident that only a very sensitive analytical method could measure the plasma concentrations of cabergoline; furthermore, this method had to be highly specific in order to distinguish the parent compound from its metabolites. A first attempt to set up a suitable RIA for cabergoline determination in plasma had proved unsuccessful (8). Following this previous experience we were able to develop suitable reagents (i.e. antibody and labelled hapten) that enabled us to

obtain a RIA with very good specificity and sensitivity. The present paper describes in detail the development of this new assay and reports the results obtained during its validation.

MATERIALS AND METHODS

Substances

Bovine Serum Albumin fraction V (BSA), caffeine, nicotine, bromocriptine, levodopa and its metabolites were from Sigma Chemical Co. St. Louis, U.S.A.; Freund's complete and incomplete adjuvants were from Difco Detroit, U.S.A.; Dextran T 10 and T 70 were from Pharmacia Uppsala, Sweden.

Cabergoline, FCE 21589, FCE 21590, FCE 21904, FCE 24592, FCE 27390, FCE 27301, FCE 27392, FCE 27393, FCE 27395, FCE 27400 and l-deprenyl were kindly provided by Farmitalia Carlo Erba CNS Department (see fig. 3 for structural formulae of cabergoline and related compounds). Benserazide was from Roche and carbidopa was a chemical reference substance from British Pharmacopoeia.

Ultima Gold liquid scintillation cocktail was from Canberra Packard, Milan, Italy.

Charcoal Norit A was from Fisher Scientific Fair Lawn, U.S.A.

All other chemicals were Farmitalia Carlo Erba analytical grade.

The 0.1 M phosphate buffer pH 7.2 was obtained as follows: 13.61 g KH_2PO_4, 30 mg Dextran T 10, 9 g NaCl, 1 g gelatine were

dissolved in 1 liter of bidistilled water and the pH was adjusted to 7.2 with 2 N NaOH.

The charcoal suspension was prepared from 2 g charcoal and 20 mg Dextran T 70 dissolved in 100 ml 0.1 M phosphate buffer, pH 7.2.

Animals

Six New Zealand rabbits weighing about 2 kg and aged two months at the beginning of the study were supplied by Charles River, Italy.

Preparation of the immunogen

Cabergoline was conjugated to BSA through the indole nitrogen via a Mannich addition involving formaldehyde condensation using the method described by Taunton-Rigby et al. (5). 100 mg of BSA are dissolved in 2 ml H_2O. To this solution 3 ml of 3 M sodium acetate and 3 ml of 37% formaldehyde are added and the resulting mixture is stirred for 5 min at room temperature. 50 mg of cabergoline added with 0.79 μCi of ^{3}H-cabergoline (obtained from the Radiochemical Department of Farmitalia Carlo Erba and prepared as described later) are dissolved in 1 ml of 1:1 (v/v) tetrahydrofurane/H_2O mixture and the resulting solution is added dropwise under stirring to the BSA solution.

The final solution is stirred at 4°C in the dark for 15 h. Then the solution is dialysed twice for 24 h against 4 l of distilled water at 4°C to remove the free hapten not covalently bound to the carrier protein.

Radiometric analyses of the content of the dialysis bag gave a molar ratio between hapten and protein of about 11:1.

Immunization

30 mg of the lyophilized immunogen are dissolved in 3 ml of saline buffer, the solution is divided in 0.5 ml aliquots then frozen at -20°C. At the immunization time a 0.5 ml aliquot of the frozen immunogen solution was thawed, homogenized with 4 ml saline and 5 ml of Freund's complete adjuvant and administered to six rabbits. Each animal received about 0.5 mg of the immunogen in 1 ml final emulsion at 20 different dorsal sites by intradermal and subcutaneous injections. Booster injections of the immunogen emulsified with Freund's incomplete adjuvant were given at four-week intervals using the same procedure. Blood was collected from the central ear vein of the animals about 10 days after each booster, allowed to clot 1 h at 37°C, then centrifuged at 1100 g for 15 min in order to separate serum.

The antiserum divided into small fractions was frozen at -20°C until use in the RIA.

Labelled hapten

FCE 24592, the 6-propargyl derivative of nor-cabergoline was catalytically reduced with tritium gas at the Radiochemical Center, Amersham, U.K. After repeated chromatographic steps (6), ^{3}H-FCE 23411, the 6-propylderivative of cabergoline was obtained with radiochemical purity of 98% in the Radiochemical Department of Farmitalia Carlo Erba. ^{3}H-FCE 23411 had a specific activity of about 70 Ci/mmol (fig. 1).

The compound stored at -20°C in acetone was stable for at least three months as assessed by radio-TLC. The same procedure was used to obtain ^{3}H-cabergoline used in the preparation of the immunogen. ^{3}H-cabergoline had, as expected, a lower specific activity than ^{3}H-FCE 23411 and for this reason the latter was choosen as a radiolabelled hapten.

Assay procedure

To obtain a standard curve, amounts of cabergoline ranging from 1.9 to 500 pg in 0.1 ml of 0.1 M phosphate buffer pH 7.2, 0.2 ml of plasma, 0.1 ml ^{3}H-FCE 23411 (about 1000 cpm) and 0.1 ml antiserum, were mixed in glass tubes in triplicate. The final volume was adjusted to 550 µl with phosphate buffer. The tubes were vortexed and left to incubate 4 h at 21°± 2°C in the dark; after 15 min cooling in an ice bath, to each tube was added 0.1 ml charcoal suspension (precooled at 4°C in an ice bath for 15 min). The charcoal suspension bound the free radiolabelled

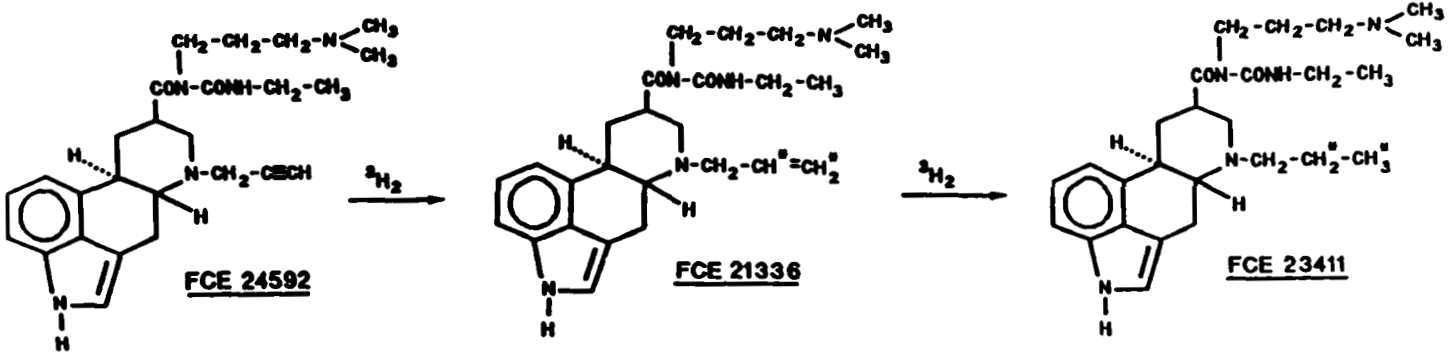

FIGURE 1. Synthesis of radiolabelled cabergoline and of the radiolabelled hapten FCE 23411 (● position of labelling).

hapten. The tubes were immediately vortexed, left at 4°C for 15 min, and centrifuged at 1200 g for 20 min at 4°C. A 0.5 ml aliquot of the supernatant of each tube containing the labelled hapten bound to the antiserum was carefully aspirated and added to vials containing 5 ml of Ultima Gold scintillation cocktail. The vials were counted in a liquid scintillation counter (counting time 20 min).

The non-specific binding (NSB) was obtained by incubating the radiolabelled hapten with 0.2 ml of plasma, with no antiserum. Standard curves in the presence of human urine were obtained by adding 25 μl of urine (from a pool of three subjects) in place of plasma and adjusting the final volume to 550 μl with buffer.

Analysis of unknown plasma and urine samples

For the analysis of cabergoline in plasma (or urine) a standard curve was set up daily in the presence of 200 μl of blank human plasma or 25 μl of human urine. Unknown samples in triplicate were incubated in the assay buffer with the antiserum and the labelled hapten and processed as described above.

Validation of the method and data analysis

All binding in the presence of standards, quality control, and unknown samples were expressed as percentage of labelled bound in the absence of ligand (Bo):

$$\% \, B/Bo = \frac{cpm\ (sample) - cpm\ (NSB)}{cpm\ (Maximum\ Binding) - cpm\ (NSB)} \times 100$$

The logit of the percentage bound was plotted against the log of cabergoline concentration and computer-fitted to a straight line. Quality control and unknown sample concentrations were calculated from the standard curve.

To determine the precision and accuracy of the assay in plasma, three quality control samples containing cabergoline at concentrations of 2.3, 31.2, and 250 pg/200 μl of plasma were analyzed in quadruplicate on seven different days over a one-month period. Quality control samples for urine were prepared at 2.9, 31.2 and 125 pg/25 μl of urine and were analyzed in triplicate on 10 different days over a two-month period.

The coefficient of variation (C.V.) of the measurements was used as index of precision. The percentage of recovered cabergoline/the amount of cabergoline added, was used as an index of accuracy.

Cross-reactivity

The ability of both known (7) and possible metabolites and other cabergoline related compounds, to compete with the tritium labelled hapten for antibody binding sites was examined using the above procedure.

We also tested the cross-reactivity of our antiserum with L-dopa and its main metabolites and with carbidopa and benserazide, which are present in commercially available L-dopa preparations. The interference given by L-deprenyl and bromocriptine was also tested since these compounds, used for the treatment of Parkinson's disease, could be co-administered with cabergoline. Finally, we tested the interference given by caffeine and nicotine since moderate coffee drinking and light cigarette smoking is usually allowed in clinical trials. Cross-reaction was defined as the amount of cabergoline causing 50% binding as a percentage of the amount of compound which produced the same binding.

Plasma samples

Blood samples were obtained from the Department of Neurology, University Clinic, Pamplona, Spain under the supervision of Dr. J.A. Obeso, Dr. G. Lera and Dr. S. Vaamonde from a 74 year old male patient with Parkinson's disease weighing 82 kg. He received daily oral doses of cabergoline as tablets, at a dose level which increased step-wise every three weeks through the following values: 3, 5 and 7 mg. The patient, who had given written informed consent, also received 750, 750 and 635 mg of L-dopa in each of three dosing regimens, respectively. Cabergoline plasma levels were monitored on the 21st day of each dosing regimen before each dose increase. Blood samples were

collected in heparinized tubes at T = 0 (immediately before cabergoline intake) and subsequently 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 hrs after the dose.

The collected blood samples were kept at 4°C until plasma was separated and stored at -20°C until examination.

RESULTS

All six rabbits given the immunogen raised antibodies against cabergoline. The best response was given by rabbit 2 after the 5th booster injection. This antiserum (C 26-2), diluted 1:4000 was able to bind about 50% of the labelled hapten added, and was therefore used in all the assays.

A linear relationship between logit % B/Bo and log of the amount of cabergoline added was found in the range 1.9-500 pg/tube. Non specific displacement of the tracer by plasma constituents was assayed by adding increasing amounts of blank human plasma or urine (pool of three subjects) in the assay. Up to 200 μl of plasma/tube could be added without any significant change of the Bo or NSB value.

The mean values obtained for Bo and NSB were 47.3±4.8 % (S.D.) and 2.3±0.8 % respectively (n = 7).

To evaluate the linearity and the reproducibility of the assay method, seven standard curves were carried out on different days in the presence of 200 μl of blank human plasma and the results averaged. The mean standard curve was defined by the

equation: logit B/B_o= -2.34 logC(pg/ml) +3.37 (r was > 0.99 in all cases) (Fig. 2a). Under equilibrium assay conditions, Scatchard analysis gave an affinity constant of 0.9×10^{11} l/mol.

The detection limit of the assay in plasma, defined as the concentration obtained from the standard curve at three times the S.D. of the B_o value was approximately 12 pg/ml.

Precision and accuracy were evaluated using samples of blank human plasma spiked with cabergoline at three different concentrations. Intra- and inter-day precision expressed as C.V. ranged from 2.9 to 6.1 % and from 6.1 to 9.8 % respectively (Table 1). Recovery ranged from 95.7 to 102.5 % (Table 1).

Up to 25 μl blank human urine/tube could be added without any significant change in the specific and non-specific binding of the ^{3}H-labelled hapten to the antiserum.

In the presence of urine, intra-assay and inter-day precision ranged from 5.4 to 10.7 % and from 12.5 to 19.0 % respectively (Table 1), with recoveries from 93.1 to 100.4 % (Table 1). The detection limit was 120 pg/ml.

The cross-reaction of the antiserum with the cabergoline related compounds tested is shown in Table 2. FCE 23411 (the compound used as radiolabelled hapten) gave the highest cross-reaction (65.2 %) among the compounds tested. FCE 21589, FCE 21904, FCE 21590, FCE 27393, FCE 27400, FCE 27390 and FCE 27391 did not cross-react. L-dopa and its metabolites: dopamine, homovanillic acid, adrenalin, nor-adrenalin, 3-oxymethyl dopa,

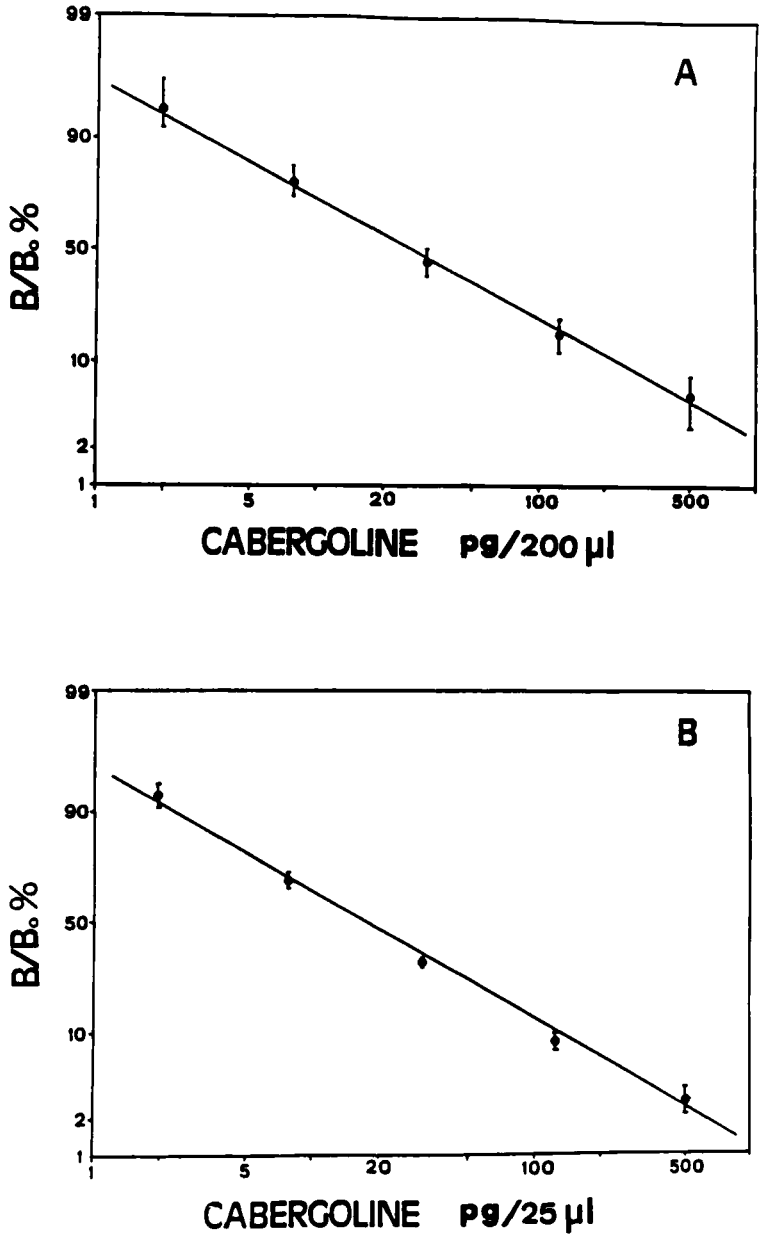

FIGURE 2. Mean standard curves obtained averaging seven standard curves from seven different days in presence of 200 µl of human plasma (A), or 25 µl of human urine (B).

TABLE 1

Accuracy and Precision of the RIA Method for the Determination of Cabergoline

PLASMA				
Amount added (pg/200 µl)	Amount found (pg/200 µl)	% Found/added	Inter-assay CV%	Intra-assay CV%
2.3 (n = 23)	2.3	100	9.8	6.1 (n=12)
31.2 (n = 28)	32.0	102.5	7.2	3.4 (n=12)
250 (n = 28)	239.3	95.7	6.1	2.9 (n=12)
URINE				
Amount added (pg/25 µl)	Amount found (pg/25 µl)	% Found/added	Inter-assay CV%	Intra-assay CV%
2.9 (n = 29)	2.7	93.1	19.0	10.7 (n=12)
31.2 (n = 30)	31.2	100.3	12.5	5.4 (n=12)
125 (n = 29)	125.5	100.4	13.7	6.4 (n=12)

TABLE 2

Cross Reactivity of some Cabergoline Related Compounds with the C 26-2 Antiserum

Compound	% Cross-reaction
FCE 21336	100
FCE 23411	65.2
FCE 27392	12.0
FCE 27395	0.75
FCE 21590	0.05
FCE 21589	< 0.01
FCE 21904	< 0.01
FCE 27390	$< 10^{-5}$
FCE 27391	$< 10^{-5}$
FCE 27393	$< 10^{-5}$
FCE 27400	$< 10^{-5}$

dihydroxyphenylacetic acid, and 3-methoxytyramine all showed cross reaction of less than 10^{-5}%. The peripheral aromatic amino acid decarboxylase inhibitors carbidopa and benserazide, the monoamino oxydase inhibitor L-deprenyl, the dopamine agonist bromocriptine, caffeine and nicotine all showed cross reaction percentages of less than 10^{-5}%. For compounds showing cross-reactivity greater than 10^{-5}% i.e. FCE 23411, FCE 27395,

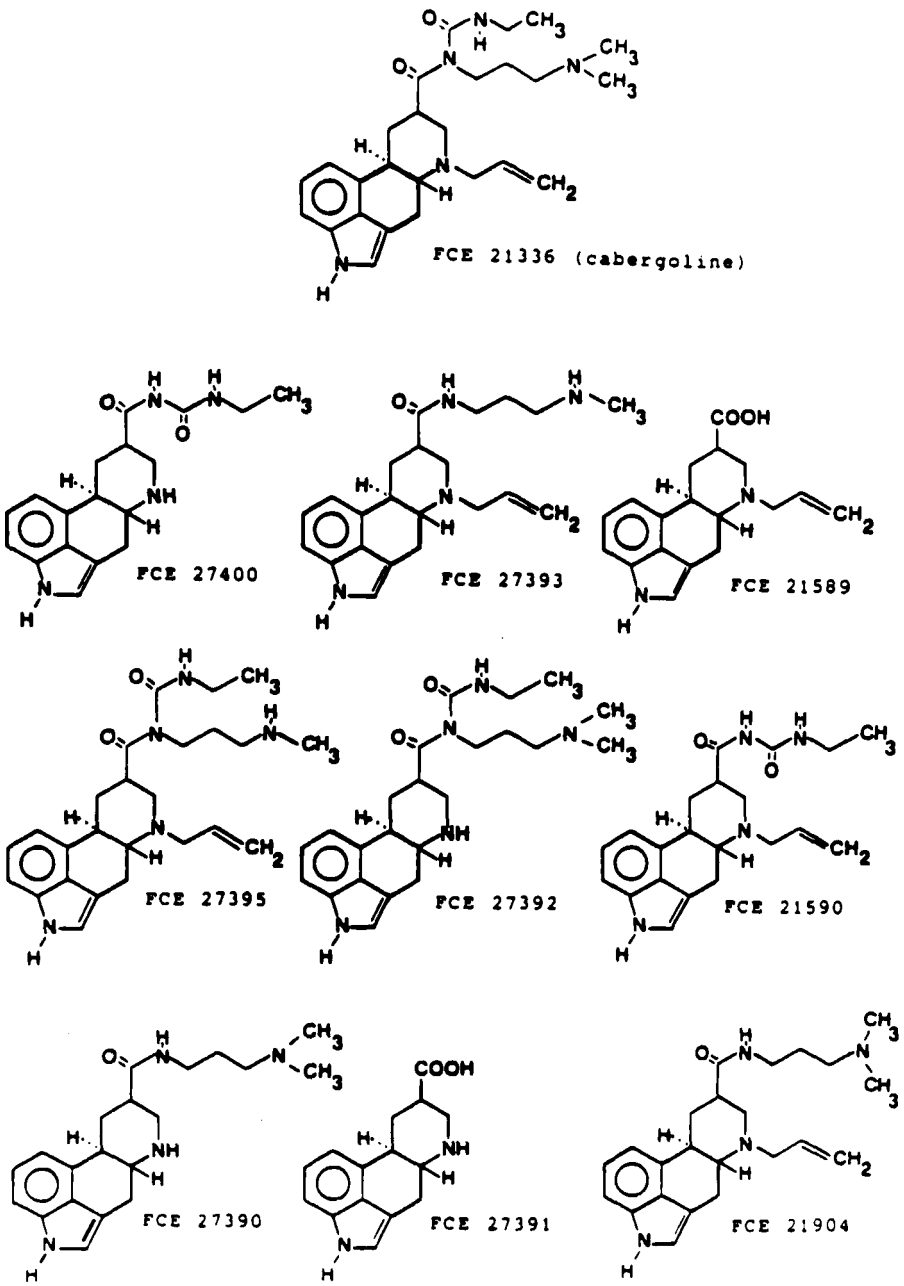

FIGURE 3. Structural formulas of the cabergoline analogs tested for cross-reactivity with the antiserum C-26-2.

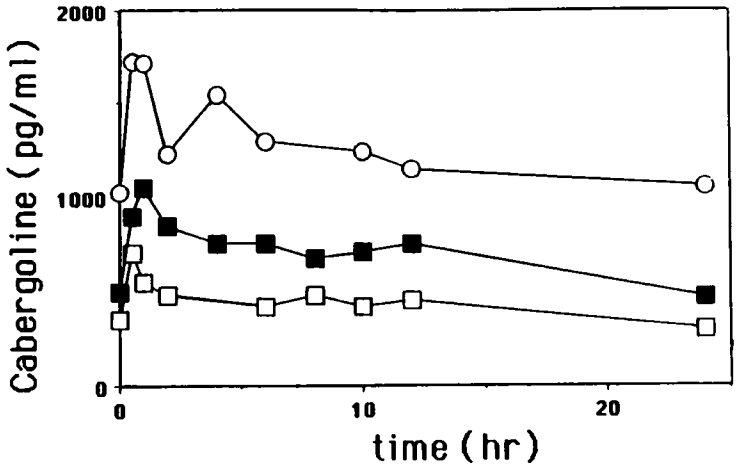

FIGURE 4. Plasma levels of cabergoline at steady state in a parkinsonian patient receiving the drug at daily oral doses of 3 (□), 5 (■) and 7 (○) mg.

FCE 21590, FCE 21589 and FCE 21904 the C.V. of cross-reaction was always below 15%.

Steady state plasma concentrations of cabergoline in a patient with Parkinson's disease who received the drug at the daily oral doses of 3, 5 and 7 mg are shown in Fig. 4. Cabergoline was absorbed rapidly with peak plasma concentrations occurring in all three sessions of treatment within 1 hr after intake. Plasma levels of the drug were related to the administered dose.

DISCUSSION

Radioimmunoassay seems to be the method of choice for the determination of dopamine-agonist compounds of high potency in

biological fluids. A common feature of this class of compounds is the need to measure very low plasma levels (in the low picogram/ml range) deriving from both the low doses administered and extensive biotransformation of the parent compounds (9). Hümpel et al. (10) described an analytical method for lisuride involving extraction of plasma followed by RIA analysis using a tritium-labelled hapten and an antiserum raised in rabbits by immunisation with lisuride-4-hemisuccinate conjugated to BSA. A similar method was developed by Krause for transuride (11). Both these authors obtained detection limits for the parent compound in the low picogram/ml range, but some interference from blank plasma was reported. In contrast we were able to add up to 200 μl of unextracted blank human plasma without any interference in the assay. Furthermore blank plasma samples assayed on different days never gave false positive results. The specificity of our antiserum against a large spectrum of possible interferents proved excellent, with the exception of FCE 27392, which showed significant cross-reactivity (12%). FCE 27392 was, however, never found in relevant amounts among cabergoline metabolites in human urine (P. Dostert, personal communication). The high sensitivity we obtained is the result of both the high affinity of the antibodies produced and the high specific activity of the label used in the assay (more than twice that reported by the two aforesaid authors). It must be stressed, however, that the increase in the specific activity did not increase the

instability of the label which could be used for several months without further purification.

The suitability of this analytical method for the determination of cabergoline in plasma was demonstrated by the results obtained in a parkinsonian patient who received the drug at the daily oral doses of 3, 5 and 7 mg. Cabergoline is rapidly absorbed since maximum plasma concentrations occurred within 1 hr in all three sessions of treatment.

This method is currently being applied to the determination of cabergoline in biological fluids from several clinical trials.

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