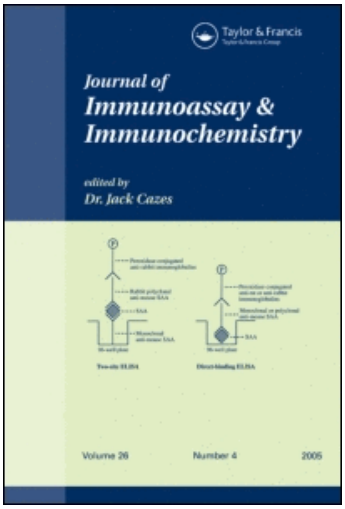

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ENZYME IMMUNOASSAYS FOR BROMOCRIPTINE AND ITS METABOLITES

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ABSTRACT

We have developed two bromocriptine enzyme immunoassays with different specificities for applications in human and animal pharmacokinetic studies. The first assay uses antibodies directed against the cyclopeptide stucture of bromocriptine, and is specific for untransformed bromocriptine. The second assay uses antibodies directed against the bromolysergic part of the molecule and allows the measurement of both bromocriptine and its metabolites. Enzymatic tracers were obtained by covalent coupling of bromocriptine analogs to acetylcholinesterase from the electric eel *Electrophorus electricus*. Both assays have a limit of detection of 10 pg/ml and a limit of quantification of 50 pg/ml. The specificity of the assays was determined following fractionation by high-performance liquid chromatography of rat samples obtained after administration of bromocriptine.
(KEY WORDS: bromocriptine, enzyme immunoassay, metabolism, pharmacokinetics)

INTRODUCTION

Bromocriptine is a dopamine D2 receptor agonist of the ergot akaloid family and is used for the treatment of Parkinson's disease (1), acromegaly (2) and hyperprolactinemic disorders (3). Orally administered bromocriptine is moderately absorbed and extensively metabolized during first-pass hepatic extraction (4,5). As a consequence, plasma levels rapidly fall below 100-200 pg/ml after therapeutic doses. Although classical physicochemical methods lack sensitivity for bromocriptine measurements in plasma (6), a mass spectrometric determination with a limit of quantification of 10 pg/ml has been described (7). However, this technique requires large sample volumes, is time-consuming, and uses equipment not available in all laboratories.

In order to measure easily and sensitively bromocriptine or other ergot alkaloids in biological fluids, our strategy, and that of other laboratories, was to use radioimmunoassays, with dihydroergocryptine labeled with iodine 125 or tritium as tracer (8-10). The difficulty of radiolabel synthesis, and the frequency with which it must be performed, are two of the drawbacks of these assays. Furthermore, metabolites interference has not been fully defined and discrepancies are observed between published plasma profiles (3,7,8-11).

Although the main pharmacokinetic properties of bromocriptine are known, there is still a need for fast, accurate and sensitive plasma measurements for pharmacological investigations, bioequivalence studies and pharmaceutical

follow-up. We are, for instance, currently investigating the reliability of pharmacokinetic parameters in bioequivalence assessment for new therapeutic formulations, and the potential pharmacological effect of bromocriptine metabolites. For this purpose, we have developed two enzyme immunoassays with a two-fold aim. The first objective was to obtain a convenient immunoassay with a detection limit of 50 pg/ml or less for specific measurement of untransformed bromocriptine. This led us to develop an enzyme immunoassay using acetylcholinesterase (AChE) as label. This enzyme has a high turnover and has been used to develop sensitive enzyme immunoassays (12,13). The second objective took into consideration recent findings showing that metabolites of orally administered drugs may reflect absorption more accurately than the parent drug (14). Since bromocriptine is a typical drug with a marked first-pass effect, we developed an enzyme immunoassay which can measure both unchanged drug and bromocriptine metabolites. The strategy of immunogen preparation and assay development was based on knowledge of the biotransformation route of bromocriptine (Figure 1).

MATERIALS and METHODS

Chemicals

Bromocriptine, dihydroergotamine, dihydroergocristine, dihydroergocornine and dihydrolysergamine were from Galena State

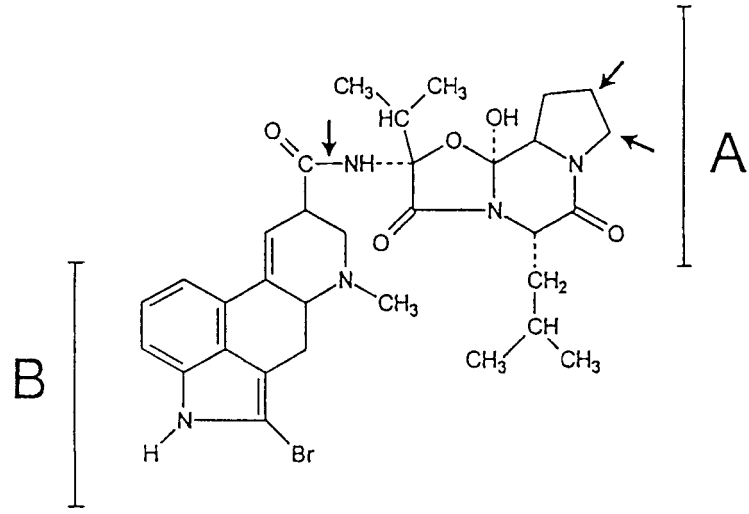

Figure 1 : Chemical structure of bromocriptine and strategy in enzyme immunoassay development. The arrows indicate the site of bromocriptine metabolism: cleavage of the amide bond and hydroxylation of the proline of the cyclopeptide structure (4,5). Part A is that recognized by the antibodies used in the bromocriptine assay. Part B is that recognized by the antibodies used in the bromocriptine metabolite assay.

Corporation (Opava, Czech Republic). Lysergic acid was from Sandoz (Rueil Malmaison, France) and dihydroergocryptine from Laboratoires Logeais (Issy, France). Dopamine and α-ergocriptine were from Sigma (Saint Louis, MO, USA), and all other ergot alkaloids used for cross-reaction studies were synthesized in our laboratory. Acetylcholinesterase (AChE) (E.C.3.1.1.7) was extracted from the electric organ of the *Electrophorus electricus* eel, and was purified by one-step affinity chromatography as described elsewhere (15). The use of the G4 form of the enzyme for the synthesis of enzymatic tracers used in

enzyme immunoassays has been patented by our laboratory. The purified enzyme is available from SPI-BIO (Massy, France). Enzyme activities were measured using Ellman's reagent, an AChE substrate comprising 2.2 g of acetylthiocholine and 1 g of dithionitrobenzene in 200 ml of 0.05 M phosphate buffer pH 7.4. One Ellman unit is defined by the concentration of enzyme producing an absorbance increase of 1 during 1 min in 1 ml of substrate medium for an optical path length of 1 cm.

Immunogen Preparation and Immunization (Antiserum Preparation)

Although the specificity of the two enzyme immunoassays was confirmed as described in the results section, the first enzyme immunoassay was designated as "bromocriptine assay", and the second as "bromocriptine metabolite assay".

Bromocriptine antiserum. Dihydroergotoxine mesylate (DHET, Figure 2A) was used as hapten as previously described (9). Briefly, DHET was covalently linked to bovine serum albumin (BSA) according to a modified Mannich's reaction involving formaldehyde as a coupling agent. After reaction, the immunogen was dialyzed and injected intradermally into five rabbits in order to induce antibody production. The selected bleeding (L116S15) was used at an initial dilution of 25,000.

Bromocriptine metabolite antiserum. Bromolysergic acid (BA, Figure 2C) was conjugated to BSA by the carbodiimide method. BA (8 mg, i.e. 23 μmol) was

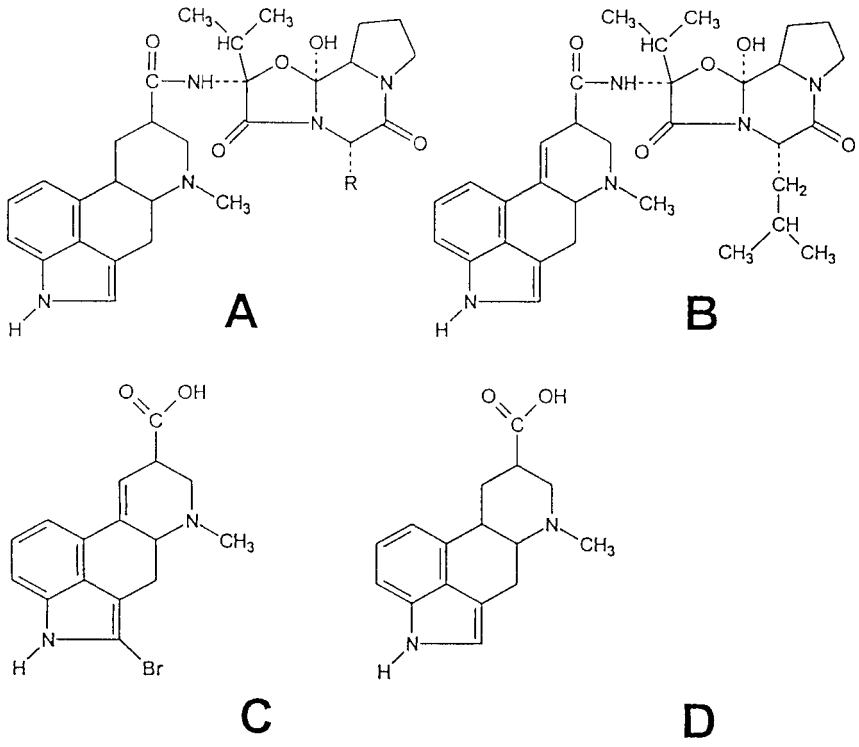

Figure 2 : Chemical structures of dihydroergotoxine (A, hapten for assay I), α-ergocryptine (B), bromolysergic acid (C, hapten for assay II) and dihydrolysergic acid (D). Dihydroergotoxine is a mixture of equal amounts of dihydroergocriptine (R = CH_2-CH-$(CH_3)_2$), dihydroergocristine (R = CH_2-phenyl) and dihydroergocornine (R = CH-$(CH_3)_2$).

dissolved in 4 ml of anhydrous dimethylformamide with continuous stirring for 5 h at 4°C in the dark. N-hydroxysuccinimide (NHS, 63 µl at 42 mg/ml) and N,N' dicyclohexyl carbodiimide (63 µl at 81 mg/ml) were added. The reaction was allowed to proceed in the dark, at 4°C, with stirring, for 48 h. BSA (50 mg) dissolved in 20 ml of borate buffer pH 8.5 (0.71 µmol) was added and the

mixture was incubated at 4°C in the dark, with stirring for 24 h. The conjugate was then dialyzed against 0.1 M phosphate buffer pH 7.4 for 48 h at 4°C. Measurement of absorbance in the dialysate indicated a bromolysergic acid/protein conjugation ratio of 12 moles of bromolysergic acid derivative per mole of BSA. Five rabbits were immunized intradermally with 1 mg of the conjugate in Freund's complete adjuvant. After 6 weeks, intradermal booster injections (0.5 mg conjugate) were given at 1-month intervals. Bleedings were made every week after the first booster injection and sera were tested for antibody binding parameters. The selected bleeding (L624S19) was used at an initial dilution of 20,000.

Enzymatic Tracer Preparation

Ergocryptine-AChE. Tracer was obtained by conjugation of α-ergocryptine (EC, Figure 2B) to acetylcholinesterase (AChE). Thiol groups were first introduced into EC by reaction of EC (9 μmol) with succinimidyl-S-acetyl-thioacetate (SATA)(28 μmol) in the presence of dimethyl-amino-pyridine (0.5 μmol) in 1 ml of tetrahydrofurane/dimethylformamide (90/10, v/v). The mixture was allowed to react for 72 h at room temperature in the dark. Activated EC was purified on a Sep-Pak column previously washed successively with 5 ml of methanol, 5 ml of 8 M urea, and 10 ml of distilled water. After application of 100 μl of the reaction mixture, the column was washed with 50 ml of distilled water and activated EC was eluted with 5 ml of

methanol/4% acetic acid (90/10; v/v). Activated EC (0.1ml) was then diluted in 0.7 ml of 0.1 M borate buffer pH 6, containing 0.5 mM EDTA. Thioester groups were hydrolyzed by addition of 0.2 ml 1 M hydroxylamine pH 7.4. Thiol-containing EC (2.5 nmol) was mixed with AChE (0.25 nmol) previously activated with N-succinimidyl-4 (N-maleimidomethyl) cyclohexane 1-carboxylate (16) and incubated overnight at 4°C. Enzymatic tracer was purified by means of molecular sieve chromatography using a Biogel A 1.5 (Biorad, Paris, France) column (90 x 1.5 cm) eluted with 0.1 M Phosphate buffer pH 7.4 containing 0.4 M NaCl, 5mM EDTA, 0.1% BSA and 0.01% sodium azide. 2 ml fractions were collected and the peak exhibiting AChE activity was collected and stored at 4°C. This enzymatic tracer was used with the bromocriptine antiserum (bleeding L116S15). After optimization of reagent concentrations, the selected activity for use in the enzyme immunoassay was 20 Ellman units.

Dihydrolysergic acid-AChE. Dihydrolysergic acid-AChE was obtained by conjugation of dihydrolysergic acid (Figure 2D) to AChE by the carbodiimide method as previously described for bromolysergic acid. Esters of NHS and dihydrolysergic acid (300 nmol) were mixed with 100 µg (0.3 nmol) of AChE in 900 µl of 0.1 M borate buffer pH 8 and incubated for 7 h at room temperature. Enzymatic tracer was purified by molecular sieve chromatography as described above for ergocryptine-AChE. This enzymatic tracer was used with the bromocriptine metabolite antiserum (bleeding L624S19). After optimization

of reagent concentrations, the selected activity for use in the enzyme immunoassay was 1 Ellman unit.

Enzyme Immunoassay

Ninety-six well microtiter plates were coated with mouse monoclonal antibodies specific for rabbit IgG (SPI-BIO, Massy, France). Before use, coated plates were washed with 0.01 M phosphate buffer pH 7.4 containing 0.05 % Tween 20 (washing buffer) using a microplate washer (300 µl/well and five wash cycles). The dilution buffer for tracer and antiserum was 0.1 M phosphate buffer pH 7.4 with 0.15 M NaCl, 5 mM EDTA, 0.1 % BSA and 0.01 % sodium azide (EIA buffer). Standard and quality control samples were diluted in drug-free human plasma (Centre National Transfusion Sanguine, Les Ulis, France). The assay was performed in a total volume of 150 µl. Reagents were dispensed as follows: 50 µl of sample, quality control or standard, and 50 µl of antiserum. To improve assay sensitivity (factor of 2), the addition of tracer (50 µl) was delayed for 24 h in both assays. After incubation at room temperature for 24 h, the plates were washed as described above and Ellman's reagent (200 µl) was dispensed into each well and incubated in the dark without agitation. When the absorbance at 414 nm in the "Bo" well (bound enzyme activity in the absence of competitor) reached 0.2-0.4 absorbance unit, the absorbance was measured in each well using a Multiskan Spectrophotometer (Labsystems, les Ulis, France). Unknown concentrations were calculated from a standard curve modeled with a

cubic spline transformation (Immunofit, Beckman, Gagny, France) for the bromocriptine assay, or with a 4-parameter logistic transformation for the bromocriptine metabolite assay. All measurements for standards and samples were made in duplicate. Non-specific binding was determined in wells in which the antiserum was replaced by 50 µl of EIA buffer.

Specificity Study

The first approach was a cross-reaction study with various ergot alkaloid derivatives and bromocriptine metabolites (8' hydroxybromocriptine and 8',9' hydroxybromocriptine). Since bromocriptine metabolites are not commercially available, they were obtained by rat liver microsomal incubations as previously described (17). Briefly, liver microsomes from rats pretreated with dexamethasone, a cytochrome *P*450 3A specific inducer, were incubated with bromocriptine and an NADPH-generating system for 30 min at 37°C. Incubation was stopped by addition of acetonitrile. Samples were centrifuged and bromocriptine related compounds were extracted from supernatants by dichloromethane. Metabolites were purified by high-performance liquid chromatography (HPLC) as described below.

The second approach was a study of immunoreactivity of rat plasma samples fractionated by HPLC. Bromocriptine was administered intravenously (1 mg/kg) and orally (10 mg/kg) to male Sprague Dawley rats. Arterial blood was collected at different times after administration. Rat plasma (20 µl) was

injected without prior extraction into a chromatographic system consisting of HPLC 510 pumps from Waters (Milford, MA, USA), an Ultrabase C18 5μm column (250 x 7.5 mm) from SFCC (Neuilly-Plaisance, France), and a fraction collector from Roucaire (Velizy-Villacoublay, France). Elution was performed at a flow rate of 1 ml/min under the following conditions: 45-minute linear gradient (0 to 100%) of solvent A (acetonitrile/ammonium carbonate 1g/l: 10/90, v/v) in solvent B (acetonitrile), followed by 10 minutes of solvent B. One-minute fractions were collected and evaporated. The dry extracts were dissolved in human plasma and assayed by each of the enzyme immunoassays.

RESULTS

For small compounds, the strategy in immunogen synthesis determines the specificity of antibodies (18). Therefore our analytical development was to obtain antibodies directed against the peptide moiety of bromocriptine (for use in the bromocriptine assay), and antibodies against the bromolysergic structure (for use in the bromocriptine metabolite assay). Different analogs of bromocriptine were studied for their cross-reactivity in the two assays (Table 1). The cross-reactivity was determined by the ratio of bromocriptine analog IC50 (concentration in pg/ml inhibiting 50% of the maximal tracer-antibody binding) to bromocriptine IC50. Although dihydroergocristine and dihydroergocornine do not share the cyclopeptide structure of bromocriptine, they were recognized in the bromocriptine assay since the antibodies used were raised against a

TABLE 1

Cross-reactivity Study

Compounds	Cross-reactivity (%)	
	I	II
Bromocriptine	100	100
Bromolisuride	<0.1	3000
Lysergic acid	<0.1	1.5
Lysergic acid amide	<0.1	3.5
Dihydrolysergic acid	<0.1	42.5
D-bromolysergic acid	<0.1	531
Dihydroergotamine	1.8	0.5
Dihydroergocristine	35	0.2
Dihydroergocryptine	810	1.1
Dihydroergocornine	249	1.8
Dihydrolysergamine	<0.1	2.8
Dopamine	<0.1	<0.1
M1/M2	2.5	120
M3	<0.1	86
M4	0.3	154

I =Bromocriptine antiserum; II = Bromocriptine metabolite antiserum

M1/M2 = 8' Hydroxybromocriptine stereoisomers

M3/M4 = 8',9' Hydroxybromocriptine stereoisomers

mixture of these compounds. Other compounds differing from bromocriptine in the cyclopeptide component (such as dihydroergotamine and hydroxylated metabolites) were unreactive or only weakly reactive with bromocriptine antibodies. Furthermore, compounds without the peptide moiety, such as bromolysergic acid or bromolisuride, were not recognized by these antibodies. In contrast, with the bromocriptine metabolite antibodies, hydroxylated metabolites M1 to M4 were significantly recognized. Since major biotransformation of bromocriptine occurs through oxidation of proline and cleavage of the amide bond (Figure 1), and according to the pattern of assay specificity, it was therefore assumed that one assay was specific for untransformed bromocriptine and the other for bromocriptine and some of its hydroxylated and bromolysergic metabolites. In order to check these assumptions, bromocriptine was administered to rats and two samples were submitted to HPLC fractionation (Figure 3A and 3B). Fractions measured by bromocriptine assay showed a single immunoreactive peak which co-migrated with an immunoreactive peak of a drug-free rat plasma spiked with bromocriptine and submitted to the same chromatographic procedure. The same fractions, when examined by the bromocriptine metabolite assay, displayed several immunoreactive peaks corresponding to untransformed bromocriptine and bromocriptine metabolites. The immunoreactive profile obtained with the

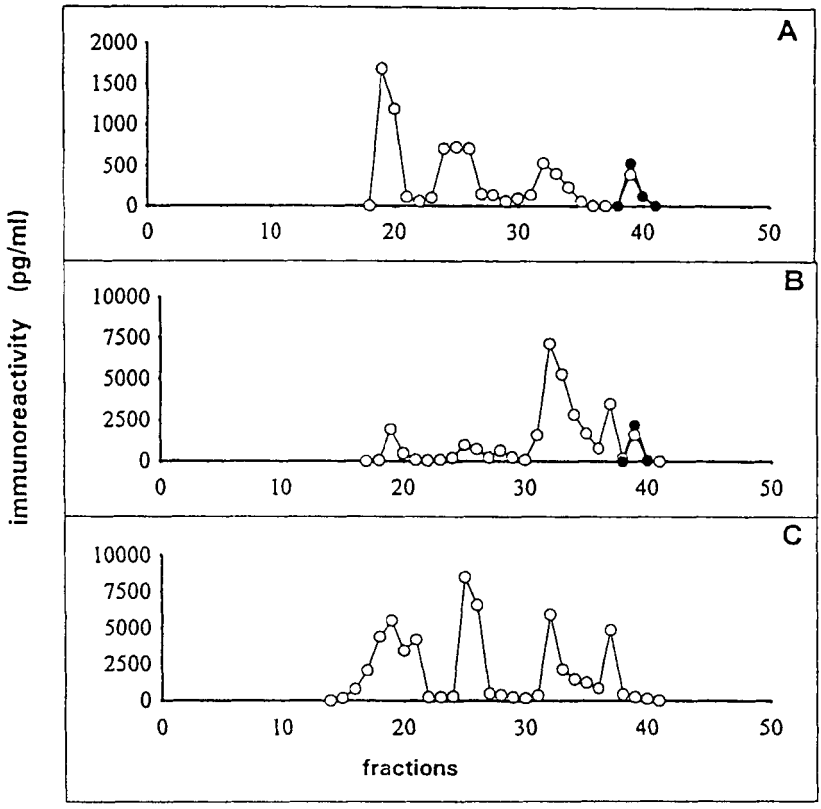

Figure 3 : Immunochromatographic profile of rat plasma taken 18 hours after oral administration of 10 mg/kg of bromocriptine (A) or 30 min after intravenous administration of 1 mg/kg of bromocriptine (B). Immunochromatographic profile (C) was obtained after incubation of bromocriptine with rat microsomes. The fractions were assayed with either the bromocriptine (•) or the bromocriptine metabolite (○) enzyme immunoassays. Internal standard consisting of bromocriptine and added to drug-free rat plasma was eluted in fractions 39 and 40. The absence of symbol indicates that no immunoreactivity was found.

bromocriptine metabolite assay was qualitatively similar to that obtained after incubation of bromocriptine with rat liver microsomes (Figure 3C). No immunoreactive peak was found for bromocriptine, since under our conditions it was completely metabolized after incubation with the rat microsomes. This suggests that the bromocriptine metabolite assay in plasma is representative of the hepatic biotransformation of bromocriptine in rats, and also in humans since the metabolism of bromocriptine is similar in both species (6).

The plasma profiles of bromocriptine and bromocriptine metabolites after oral (PO) or intravenous (IV) administration of bromocriptine to rats are shown in Figure 4. The extent of the hepatic first-pass effect is reflected by the largest area under the curve obtained with the metabolite assay. The metabolite/untransformed bromocriptine ratios for area under the curves were approximately 10 and 100 after IV and PO administration, respectively.

Further validations were performed by studying the repeatability (intra-assay variation) and the reproducibility (inter-assay variation) for both assays (Table 2). All data were obtained with standard curves and quality controls consisting of drug-free human plasma spiked with bromocriptine. The limit of quantification corresponding to the lowest concentration measured with satisfactory precision (repeatability and reproducibility) and accuracy (19) was 50 pg/ml for both assays. The minimal detectable dose (usually three standard

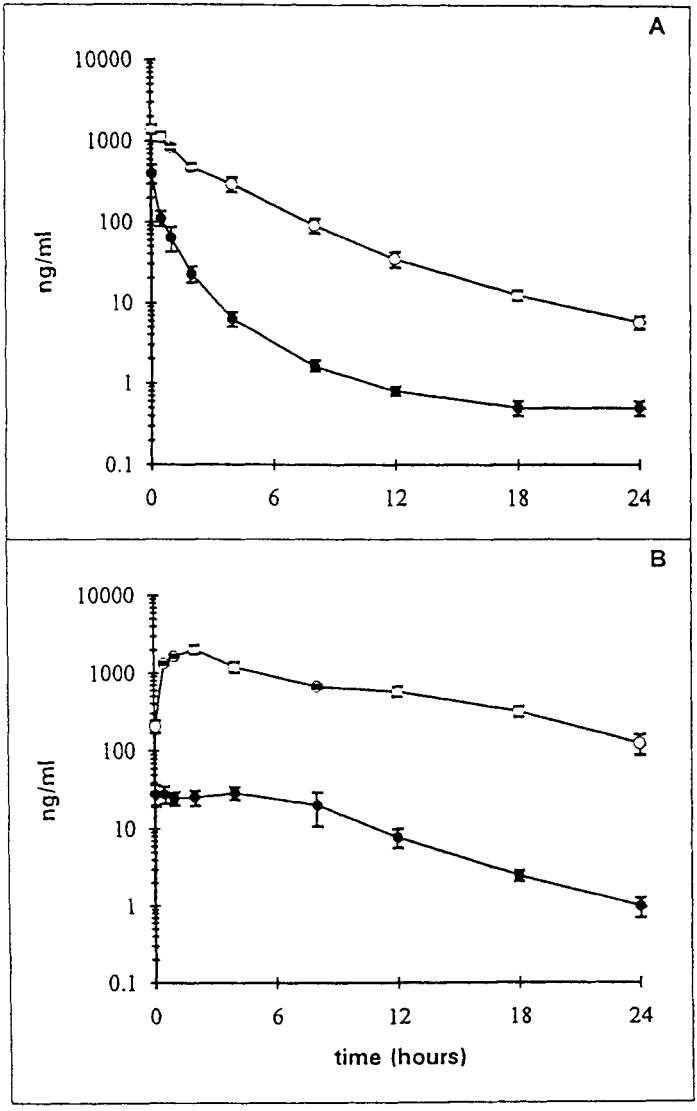

Figure 4 : Bromocriptine (•, bromocriptine assay) or metabolites (o, bromocriptine metabolite assay) profiles after intravenous administration of 1 mg/kg (A) or oral administration of 10 mg/kg (B) of bromocriptine. The means ± SEM for 6 rats are shown.

TABLE 2

Accuracy and Reproducibility of the Enzyme Immunoassays

	Bromocriptine added (pg/ml)	Bromocriptine recovered (pg/ml)	% accuracy	CV, % (intra-assay)	CV, % (inter-assay)
I					
	1000	1264	26.4	26	27
	600	661	10.2	8.8	10.7
	200	232	16	17.1	9.5
	100	119	18.7	11.5	5.8
	50	41	18	24	17.7
	20	12	-40	27	42
II					
	1000	1009	0.9	3.6	6.5
	600	639	6.5	4.8	8.9
	200	215	7.5	5.2	10.5
	100	110	10	11	12.8
	50	48	-4	19.7	21
	20	24	20	43.3	37

I = Bromocriptine assay; II = Bromocriptine metabolite assay

intra-assay : n=5; inter-assay : n = 5

deviations from the response without competitor) was 10 pg/ml for both assays. Typical standard curves for both assays are presented in Figure 5.

DISCUSSION

This study demonstrates that the specificity of antibodies for bromocriptine may be orientated in order to develop immunoassays of either untransformed bromocriptine (bromocriptine assay) or of a pool of metabolites plus untransformed bromocriptine (bromocriptine metabolite assay). The strategy of immunogen preparation was based on knowledge of the major pathways of bromocriptine metabolism (4,5). Since bromocriptine is metabolized by hydroxylation of the cyclopeptide bond and/or cleavage of the amide bond between the lysergic group and the cyclopeptide ring, it was suspected that antibodies directed against bromolysergic acid were able to recognize bromocriptine and metabolites. Inversely, antibodies directed towards the cyclopeptide ring might not be able to recognize metabolites. This was confirmed by the cross-reactivity study and the assay of rat plasma samples after HPLC fractionation. Although the 8' hydroxylated metabolite cross-reacted weakly in the bromocriptine assay, its contribution to the total immunoreactivity was negligible as was seen in the immunochromatograms. Besides its specificity, the bromocriptine enzyme immunoassay appears more convenient and sensitive than previous specific radioimmunoassays and may

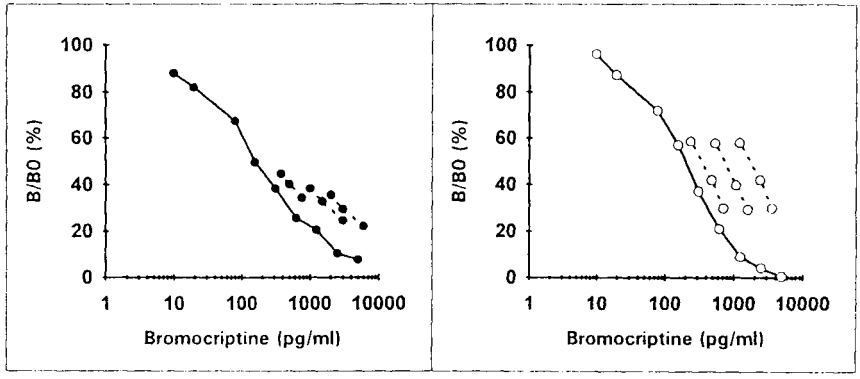

Figure 5 : Standard curves (solid line) and serial dilutions of rat samples (dotted line) for the bromocriptine (•) and the bromocriptine metabolite (o) enzyme immunoassays.

enable longer monitoring of bromocriptine in plasma after clinical dosing in humans.

As bromocriptine has a large spectrum of established and potential therapeutic applications, it is subject to continuous biopharmaceutical and pharmacological development, for which pharmacokinetic investigations are required. Recent examples include the bioequivalence studies of new formulations (20,21), the testing of different routes of administration (11,22), and the study of the influence of the physiological state (23). In the field of bioequivalence evaluations, our present enzyme immunoassays may help decision-making in reducing the intra- and inter-subject pharmacokinetic variability usually encountered with drugs with a high first-pass effect, such as

bromocriptine and other ergot alkaloid derivatives. As a matter of fact, one hypothesis in bioequivalence assessment is that the pharmacokinetic characteristics of the parent compound plus a pool of metabolites are more stable than those of the parent drug alone, and that the biotransformation products can be used on a "pick out the best" basis for statistical assessment of bioequivalence (14). Therefore, our strategy in bioequivalence studies involving ergot alkaloids is to help decision-making by measuring both unchanged drug and its metabolites.

Besides the pharmacokinetic argument, the difficulties in assessing low plasma concentrations of the parent drug may be circumvented by the measurement of highly concentrated metabolites. It should be noted that nothing is known about the respective quantities of bromocriptine metabolites. Hence, one criticism of our analytical approach may be that the enzyme immunoassay likely measures a pool of metabolites rather than a single compound. There is no way of circumventing this lack of metabolite discrimination. Increased specificity between metabolites could be achieved by chromatographic sample fractionation and measurements of separated metabolites. In our experience, this approach decreases assay sensitivity and reproducibility and is not easily applicable to a large number of samples.

Another point which is still controversial is that the pharmacological activity of metabolites is as yet unproven (24,25). Using the present enzyme

immunoassays, we are currently addressing this problem. Following different routes of administration we can demonstrate, as shown in figure 4, different ratios of metabolite/parent drug concentrations and correlate them with a pharmacological effect, such as the lowering of circulating prolactin, a typical action of dopamine D2 agonists. The overall goal is to integrate pharmacokinetic and pharmacodynamic data to produce mathematical models allowing metabolite activity to be inferred. Such an approach has already been successfully used for other drugs (26).

In conclusion, these new analytical tools may improve knowledge of bromocriptine pharmacokinetics and pharmacodynamics and offer a good model for other compounds of the ergot alkaloid family.

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