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Immunoassays for the detection of nicergoline and its metabolites in human plasma

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Abstract

In order to determine nicergoline pharmacokinetics after oral administration to humans, we have developed two radioimmunoassays, one directed against nicergoline and the other directed against known nicergoline metabolites. The assays were validated according to the recommendations of international regulatory agencies and their limits of quantification were 40 and 10 pg/ml, respectively. In order to further validate the methods, a chromatographic separation of immunoreactive entities was performed with samples from healthy volunteers who were given 15 mg of Sermion® (nicergoline orally administered). Chromatographic determination of assay specificity showed that the metabolite radioimmunoassay recognised known nicergoline metabolites but also a new metabolite. Using the antibodies directed against nicergoline, we were unable to detect nicergoline in the human plasma. This suggests that nicergoline is absent in the circulation because of complete metabolism through its first-pass effect.

Keywords: Nicergoline; Radioimmunoassay; Metabolite; Pharmacokinetics

1. Introduction

Nicergoline (10α-methoxy-1,6-dimethylergoline 8β-methanol-5-bromonicotinic acid (Fig. 1) is an alpha-adrenergic blocking agent whose cerebrovascular vasodilator activities contribute to enhancement of cerebral blood flow and metabolism in humans. Nicergoline improves vigilance and information processing at the neurophysiological level, which leads to clinical improvement both in degenerative and vascular dementia [1]. This potential clinical effect has been confirmed recently in a multicentre, randomised, double-blind study of the efficacy and safety of the drug in patients with multi-infarct dementia [2]. In France, nicergoline is used for the treatment of senile mental impairment in elderly subjects and ranks among the most administered drugs.

After oral administration, nicergoline is extensively metabolised into 1-methyl-10α-methoxy-

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9,10 dihydrolysergol (MMDL), 1-hydroxymethyl-10α-methoxy-9,10 dihydrolysergol (1-OHMDL) and 10α-methoxy-9,10 dihydrolysergol (MDL) (Fig. 1). Pharmacokinetic studies of nicergoline have been based on the determination of these metabolites, for which an HPLC method coupled to mass spectrometry has been developed [3]. High inter-subject variability was observed [4] and this may be attributed to metabolic polymorphism since it has recently been shown that conversion of MMDL into MDL is catalysed to a major extent by cytochrome 2D6 and that the observed interindividual variation in the metabolic pattern is related to the polymorphism of debrosiquine, a known substrate of cytochrome 2D6 [5].

One paradox is that nicergoline concentrations have never been determined in biological fluids nor at its sites of action by conventional methods. Attempts have been made to develop specific measurements of nicergoline in biological fluids, but no drug pharmacokinetics have been reported in animals or humans since nicergoline was below the limit of quantification of reported analytical methods [6,7]. Therefore, the aim of this study was to develop a sensitive analytical method for nicergoline and its metabolites in order to assay circulating nicergoline and determine its kinetics. Immunoassays have limits of detection one order of magnitude better than those of conventional methods and were chosen for development of two radioimmunoassays, the first intended to be specific only for nicergoline and the second for the metabolites lacking the bromonicotinic moiety of nicergoline. In this report, we show that nicergoline is highly metabolised and undetectable in the circulation.

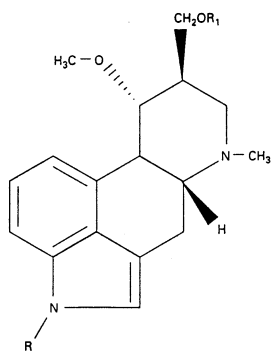
2. Experimental

2.1. Chemicals

Nicergoline, *N*-demethyl-nicergoline, MDL, 1-OHMDL and MMDL were from Galena State Corporation (Opava, Czech Republic). Sodium iodine 125 was from Amersham (Les Ulis, France). Standard chemicals were from Sigma (Darmstadt, Germany) or Merck (St Louis, MO). Immunisations were done in Blanc du Bouscat male rabbits (Evic, Roquefort, France).

2.2. Immunogen preparation and immunisation

Antibodies to nicergoline were obtained as follows: *N*-demethyl-nicergoline (Fig. 1) was used as a hapten and was covalently linked to bovine serum albumin (BSA) using formaldehyde as a coupling agent. While continuously stirring, 4 ml of a 37% aqueous solution of formaldehyde was added to 210 mg of bovine serum albumin (BSA) in 3 ml of 2 M sodium acetate. After 5 min, 25 mg of *N*-demethyl-nicergoline dissolved in 1 ml of 2 M acetic acid was added and the mixture was



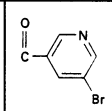
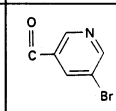
	R1	R
Nicergoline		CH_3
N-dem-nicergoline		H
MMDL	H	CH_3
MDL	H	H
1-OH-MDL	H	OH

Fig. 1. Structure of nicergoline, *N*-demethyl-nicergoline and its metabolites MMDL, 1-OH-MMDL and MDL.

stirred for 24 h at room temperature in the dark. The conjugate was dialysed against distilled water at 4°C over 4 days. Spectrophotometric measurement of uncoupled *N*-demethyl-nicergoline in the dialysate indicated that 11 moles of hapten were coupled per mole of BSA. One mg of conjugate mixed with an equal volume of complete Freund's adjuvant was injected intradermally into five rabbits in order to induce antibody production. Booster immunisations were repeated every 6 weeks. The selected bleeding (L400S20) was used at an initial dilution of 25 000. The same bleeding was used in the immunoassay for nicergoline metabolites.

2.3. Iodinated tracer synthesis

N-demethyl-nicergoline and MDL were iodinated according to the chloramine T method [8]. To 5 μl of *N*-demethyl-nicergoline or MDL (1 mg/ml in methanol), were successively added 20 μl of phosphate buffer (pH 7.4; 0.5 M), 5 μl of $Na^{125}I$ (180 Mbq) and 5 μl of chloramine T (3 mg/ml in water). After 1 min of incubation with stirring, 5 μl of sodium metabisulfite (15 mg/ml in water) was added to stop the reaction. The mixture was applied to thin layer chromatography and the chromatogram was developed in chloroform–methanol (80:20, v/v). The radioactive bands corresponding to radioiodinated derivatives were revealed by autoradiography and eluted with methanol after transfer of the silica to a Pasteur pipette. The radioactive tracers were then stored at −80°C. These tracers were used for the development of two radioimmunoassays: ^{125}I-*N*-demethyl-nicergoline was used to develop an assay supposed to be specific for nicergoline and ^{125}I-MDL for the nicergoline metabolite assay.

2.4. Radioimmunoassay (RIA) procedure

The diluent buffer used was phosphate buffer (pH 6.5; 0.1 M) containing 0.1% bovine serum albumin, 0.15 M NaCl and 0.01% sodium azide. Standards were prepared in human plasma free (Etablissements de Transfusion Sanguine, Rungis, France) of nicergoline or nicergoline metabolites from stock concentrations at 40 ng/ml. RIAs were performed in polystyrene tubes using a total volume of 0.3 ml. Standard or sample (0.1 ml) was mixed with 200 Bq of iodinated tracers (^{125}I-*N*-demethyl-nicergoline for the nicergoline RIA or ^{125}I-MDL for the nicergoline metabolite RIA) and 0.1 ml of diluted antiserum. After incubation for 16–18 h at 4°C, bound tracer was separated from free tracer by adding 0.1 ml of pig anti-rabbit immunoglobulins (referenced pool/8 and prepared in our laboratory) and 1 ml of 6% polyethylene glycol. After 30 min of incubation at 4°C, the tubes were centrifuged at 3500 rpm for 25 min at 4°C. After removal of the supernatant, the pellet was counted in a gammacounter (LKB rackbeta from Pharmacia). Unknown concentrations were calculated from a standard curve linearised using a 4-parameter logistic model (Immunofit from Beckman, Gagny, France). Three quality control samples in duplicate were introduced in order to control assay accuracy and reproducibility.

2.5. Assay validation

Both RIAs were validated according to international recommendations [9]. The stability of nicergoline or MDL in blood or plasma was assessed by incubating known concentrations for various times at different temperatures. Accuracy, repeatability and reproducibility were measured by assaying four quality control samples during the same day ($n = 6$) and on different days ($n = 6$). The limit of quantification was the lowest concentration providing satisfactory accuracy (recovery of known concentration in the range of 80–120%) and reproducibility (coefficient of variation below 20%). Cross-reactivity studies were performed by assaying known concentrations of nicergoline analogues. The percentage of cross-reactivity was defined by the ratio of the IC50 of the tested compound to that of the reference compound, multiplied by 100, where IC50 is the concentration inhibiting 50% of the radioactive tracer bound to the antibodies in the absence of competitor. The specificity was determined by fractionation of a pool of human plasma samples by HPLC and measurement of the immunoreactivity in the collected fractions. Human samples obtained at selected times after administration of

Table 1
Cross-reactivity study[a]

	Nicergoline RIA[b]	Metabolite RIA[c]
Nicergoline	100	5
N-demethyl nicergoline	100	7
MDL	<0.01	100
MMDL	0.025	92
1-OHMMDL	0.03	65

[a] Percentage of recognition (reference: nicergoline for the nicergoline RIA and MDL for the metabolite RIA).
[b] Tracer: ^{125}I-*N*-demethyl-nicergoline.
[c] Tracer: ^{125}I-MDL.

nicergoline were pooled and submitted to chromatography in a system consisting of an HPLC 510 from Waters (Milford, MA), an S5 nitril cyanopropyl column (250 × 4.6 mm) from Touzard and Matignon (France), and a fraction collector from Roucaire (Velizy-Villacoublay, France). For the determination of nicergoline metabolites, 20 μl of pooled plasma was injected directly into the chromatographic system. For the determination of nicergoline, the pooled sample (3 ml) was extracted for 1 h with 27 ml of a mixture consisting of chloroform/saturated ammonium acetate/NaOH 2 M (90:5:5, v/v/v) and centrifuged for 5 min at 3000 rpm. The organic phase was removed and dried under a continuous flow of nitrogen. The dry extract was dissolved in 0.1 ml of the mobile phase of the chromatographic system and injected into the column. Elution was performed at a flow rate of 1 ml/min with 10 mM ammonium acetate–acetonitrile (10:90, v/v). One-minute fractions were collected and evaporated. The dry extracts were dissolved in human plasma and assayed by each of the RIAs.

2.6. Clinical study

A pilot study was performed with four healthy male volunteers (age 27 ± 4 years, weight 73 ± 3 kg, height 181 ± 3 cm). All subjects were in good health as determined by clinical history, physical examination and routine clinical laboratory tests. None had received any medication during the 2 weeks before participation in the study. The subjects had a regular diet and did not smoke or drink alcoholic beverages during the study. The protocol was approved by an Ethical Review Committee and written informed consent was obtained from each subject. Sermion® was given at a dose of 15 mg and blood samples were collected in heparinised vacuum tubes at 0 (before administration), and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 9, 12, 24 and 36 h after administration. Plasma was immediately separated by centrifugation at 4°C and kept frozen at −20°C until analysis.

3. Results

The initial aim was to obtain antibodies specific to nicergoline and others specific to nicergoline metabolites. Since for small compounds the strategy in immunogen synthesis determines the specificity of antibodies, the immunogen was prepared by coupling nicergoline through its ergoline moiety in order to obtain antibodies recognising the bromonicotinic part of the compound. However, it was anticipated that the conjugated nicergoline would be susceptible to hydrolysis by esterase action leading to a conjugate of MDL and bovine serum albumin able to induce antibodies against cleavage products of nicergoline. In order to check this hypothesis, two radioiodinated tracers were synthesised allowing two radioimmunoassays for which the cross-reactivity of nicergoline and nicergoline metabolites were tested. As shown in Table 1, these tracers, namely ^{125}I-*N*-demethyl-nicergoline and ^{125}I-MDL, allowed development of two radioimmunoassays with different specificity patterns. The first tracer theoretically enabled specific assay of nicergoline in which known nicergoline metabolites were not recognised, while the second allowed measurement of metabolites. For the first radioimmunoassay, *N*-demethyl-nicergoline was recognised to the same extent as nicergoline since this compound was used for immunogen and tracer synthesis. Other ergot alkaloids possessing a structure unrelated to nicergoline were not recognised by either assay (not shown). The stan-

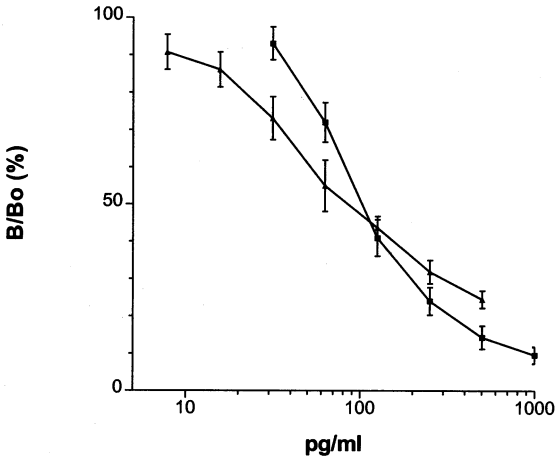


Fig. 2. Standard curves for the radioimmunoassays of nicergoline (square) and nicergoline metabolites (triangle). Each point is the mean ± SD of eight independent experiments.

dard curves for each assay are presented in Fig. 2. The two radioimmunoassays were then validated according to international recommendations. The accuracy, repeatability (intra-day variation) and reproducibility (inter-day variation) of the radioimmunoassays are presented Table 2. The limits of quantification were 40 and 10 pg/ml, respectively for nicergoline and nicergoline metabolites. Since the stability of nicergoline in blood or plasma samples was found to be less than 2 h at room temperature, all samples were processed at 4°C and stored immediately at −20°C.

The radioimmunoassays were then used for measurement of nicergoline and nicergoline metabolites in samples of subjects given a single

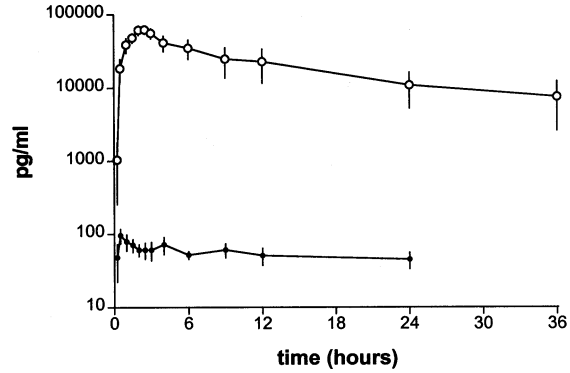


Fig. 3. Profile of mean concentrations (mean ± SE; $n = 4$) after oral administration of 15 mg of nicergoline (Sermion®): measured with the radioimmunoassays for nicergoline (closed circles) and for nicergoline metabolites (open circles).

oral doses of nicergoline. The immunoreactivity profiles are presented in Fig. 3.

In order to determine if the plasma concentrations supposed to correspond to nicergoline could be attributed specifically to the unchanged compound, the immunoreactivities detected in plasma samples were identified by HPLC coupled to radioimmunoassay of the collected fractions. The chromatographic system was calibrated by measurement of the retention time of blank human plasma samples spiked with nicergoline and its analogues and analysed using the same conditions as the plasma samples from the clinical study. The immunoreactive profile of pooled human plasma sampled 30 min after administration and measured with the nicergoline radioimmunoassay is presented in Fig. 4A. Two immunoreactive peaks

Table 2
Validation parameters

	Nicergoline RIA				Metabolites RIA			
(pg/ml)	500	100	40	20	200	50	20	10
Accuracy[a]	105	95	84	122	94	87	86	96
Repeatability[b]	10.6	2.5	8.5	3.6	6.1	6.3	6.4	7.7
Reproducibility[c]	11.4	12.4	12.4	37.7	6.4	9	15.5	15.3

[a] % recovery ($n = 6$).
[b] Intra-day % coefficient of variation ($n = 6$).
[c] Inter-day % coefficient of variation ($n = 6$).

were obtained and their retention times were shorter than those of nicergoline and *N*-demethyl-nicergoline. These immunoreactivities were defined as nicergoline-like compounds. The profile of metabolite immunoreactivity presented in Fig. 4B showed that the pool of human plasma obtained 2 h after administration produced four immunoreactive peaks. The first peak represented 25% of the total immunoreactivity and was eluted in the void volume. The others were identified by the identity of their retention time with those of the three known metabolites, i.e. MDL, MMDL and 1-1-OHMDL, and their relative proportions were 65/30/5. In order to check if the first peak was a glucuro- or sulpho-conjugate of one of these metabolites, the pool of human plasma was treated at 37°C with or without a mixture of glucuronidase and sulphoconjugase (125 and 1200 IU/ml, respectively). Independently of the enzymatic treatment, this led to the disappearance of this immunoreactive peak and to increase in the peak corresponding to MMDL (not shown).

4. Discussion

Despite nicergoline's widespread use in clinical practice, its pharmacokinetic properties have never been fully demonstrated in humans. Initial studies based on the administration of tritiated nicergoline to various animal species indicated that a small fraction of unconverted radioactive nicergoline could be detected in urine but was absent in the circulation [10]. Chromatographic methods coupled to mass spectrometry are insufficiently sensitive to detect nicergoline in human plasma and have been used only for testing the purity of nicergoline preparations [11]. More sensitive techniques such as immunoassays using antibodies directed towards the bromonicotinic moiety of the drug have been described but not applied to clinical samples [6,7]. The need in nicergoline measurement for a bioequivalence study and our experience in immunoassay for ergot alkaloids incited us to initiate a further attempt in the development of a new immunological technique for nicergoline and its known metabolites. Partial degradation of the nicergoline analogue during the immunisation process results in antisera containing antibodies specific for nicergoline and metabolites. Antibodies were selected by the use of radioiodinated *N*-demethyl nicergoline for the specific nicergoline immunoassay and radioiodinated MDL for the metabolite immunoassay.

In order to further demonstrate the specificity of these assays, a chromatographic separation was performed. Fractionation of the immunoreactivity corresponding to known nicergoline metabolites showed that at least four compounds were recog-

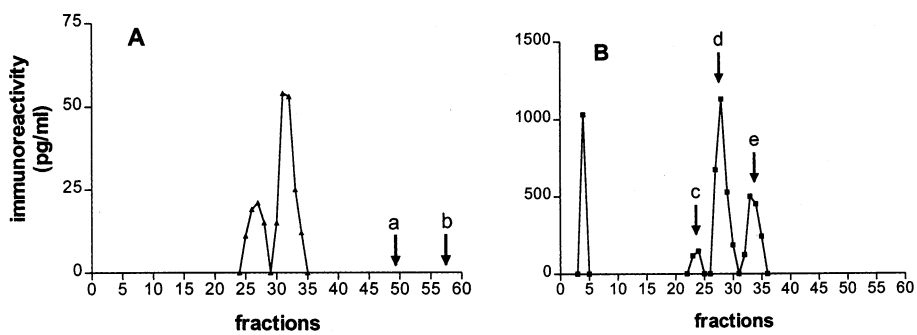


Fig. 4. Immunochromatographic profile of a pool of human samples (A) taken 0.5 h after oral administration of 15 mg of nicergoline and assayed with the nicergoline radioimmunoassay; and (B) taken 2 h after oral administration of 15 mg of nicergoline and assayed with the metabolites radioimmunoassay. The arrows indicate the elution position of synthetic nicergoline or analogues chromatographed under the same conditions: (a): *N*-demethyl-nicergoline; (b): nicergoline; (c): -OH-MMDL; (d): MDL; and (e): MMDL.

nised. The identification and proportion of the first group consisting of MMDL, MDL and 1-OHMDL were consistent with literature data [3]. A fourth structure possessing more polar characteristics was also found at high concentration. The identification of this metabolite was not in the scope of the study. It is likely that this metabolite has never been identified or measured with conventional chromatographic analyses since these techniques require an initial extraction step with chloroform which can potentially remove polar structures. Thus and although this metabolite was heat-denatured in plasma in the absence of deconjugation enzymes, it could not be excluded that it consists of glucuronic acid conjugated to one of the nicergoline metabolites. After administration of radioactive nicergoline, glucuronic acid of the primary alcohol of MMDL has been reported to be present at high concentration in monkey but not in human urine [10]. Nevertheless, and owing to the specificity of the assay, it might be speculated that conjugates at the alcoholic part of MMDL could not be recognised by the antibodies.

When the nicergoline immunoreactivity was measured after chromatography, no immunoreactivity was found at the retention time of nicergoline. Unexpectedly, the chromatogram revealed two immunoreactive peaks whose retention times were different from those of nicergoline and *N*-demethyl nicergoline. Their retention times are also apparently different from those of known nicergoline metabolites, but it can not be excluded that they correspond to a cross-reaction. Since these compounds were recognised by the antibodies, we supposed that they should possess the bromonicotinic moiety of nicergoline. This suggests that nicergoline is absent in the circulation because of complete metabolism through its first-pass effect. This does not contradict the observation that only 6–7% of radiolabelled nicergoline could be determined in an unconverted form in human urine, since in that study nicergoline was administered intravenously [10]. Low concentrations or absence of parent compounds have also been noted for other ergot alkaloids such as dihydroergocryptine [12] and dihydroergotamine [13], for which oral administration leads to undetectable parent drug or to concentrations below 100 pg/ml. For some of these compounds, hepatic metabolism leads to oxidised and hydrolysed compounds of pharmacological potency at least equal to that of the parent compound [14].

It could therefore be argued that nicergoline is a prodrug and that only its metabolites exert a pharmacological action after oral administration. This may be supported by published studies showing that in various animal models the pharmacological effects of nicergoline are due to nicergoline per se and in part to its metabolites [15]. Furthermore, Moretti et al have also demonstrated that MDL and MMDL may bind to alpa-1 receptors but with an affinity lower than that of nicergoline, and their activities were pronounced in old animals [16].

In conclusion the present study has provided new insight into nicergoline metabolism in humans through the development of specifically directed antibody probes. Although the developed assays were able to quantify nicergoline at the picogram level, we demonstrated that nicergoline was undetectable in the circulation.

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