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Radioimmunoassay of Nicergoline in Biological Material

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Abstract. A method for radioimmunoassay of nicergoline (Sermion) in plasma and urine is described. The antiserum was produced in rabbits by administration of an immunogen obtained by coupling bovine serum albumin to the nicergoline molecule at the indole nitrogen. The resulting antibodies reacted well with nicergoline and the 1-demethyl derivative and did not cross-react with the principal metabolites of these substances nor with rye ergot derivatives, in particular dihydroergotamine, methysergide and bromocriptine. The tracer was nicergoline labelled with iodine-125. The assay was sensitive because concentrations as low as 125×10^{-12} mol/l nicergoline could be directly determined in plasma and urine without prior extraction. The marked specificity and high sensitivity allowed easy determination of plasma and urine levels of this drug following administration in man.

Introduction

Nicergoline (10-methyl-1,6-dimethylergoline-8β-methanol 5-bromonicotinate) (Fig. 1) has pharmacological properties that have been applied in clinical practice for several years; this α-adrenolytic medication has selective cerebrovascular properties and consequently affects the metabolism of cerebral cells; in addition, it has a definite platelet anti-aggregating effect. Although the pharmacological properties are now becoming more clearly defined, not enough information is yet available concerning the biotransformation of this product because, until now, the existing assay methods were not sufficiently sensitive and specific to evaluate the levels of this drug in the plasma and urine. Arcamone et al. [1] conducted valuable studies on the absorption, distribution, biotransformation and excretion of tritium-labelled nicergoline; following administration to humans and to selected animal species, these authors measured the radioactivity in blood, urine and some tissues. This procedure, which was also followed by Eckert et al. [2], lacked sensitivity and, above all, specificity; it sometimes gave rise to 'delicate' interpretations. We set out to develop a radioimmunological method of assaying nicergoline in plasma and urine taking inspiration from studies conducted on other rye ergot derivatives [6, 7]. A biopharmaceutical study of nicergoline in man is reported here.

Materials and Methods

1. Reagents

Buffer used in the assay (B1) was 100 mM phosphate in 0.15 mol/l saline, pH 6.9, containing 0.1% sodium azide and 0.25% bovine serum albumine (Armour). Buffer used in labelling nicergoline (B2) was 0.1 M phosphate.

Nicergoline was supplied by Laboratoire Specia (16 rue Clisson, 75646 PARIS Cédex 13). A stock solution was prepared containing 325 mg nicergoline/l ethanol (66 µmol). This solution was stored at 4° C. Immediately before the assay, the stock solution was diluted with B1 to give standard solutions containing 2,000, 1,000, 500, 250, 125, 60 and 30 ng/l respectively.

Dextran-coated charcoal suspension in B1 was made from 0.25 g charcoal Norit (Prolabo 26008)/100 ml and 0.025 g dextran T70 (Pharmacia)/100 ml.

2. Labelled nicergoline production

The labelling of nicergoline was performed at room temperature using a technique derived from that of Greenwood [5]; 0.5 mCi (5 µl) ^{125}I (Radiochemical Centre, Amersham) was reacted with 3.5 µg nicergoline (in 20 µl) and 80 µg chloramine T (Prolabo 22 685 265) (in 20 µl). After 1 min under agitation 100 µg (in 20 µl) sodium metabisulphate (Prolabo 27 921 298) was added to stop the oxidation. After addition of 500 µg potassium iodide (Prolabo 26 846 23) in 50 µl the mixture was transferred on to silica gel (Merck Kieselgel 60). The chromatogram was developed in chloroform + methanol + water solvent (90:10:1). The pure product (Rf 0.6) was isolated from the radioactive impurities by radiochromatography and extracted with ethanol. The resulting solution was stored at −20° C. Immediately before use this solution was diluted with B1 to obtain approximately 15000 cpm in 100 µl solution. The specific activity was calculated as a function of the yield together with the initial amounts of isotope and unlabelled peptide. This calculation was verified by adding two, three and four times the amount of tracer as unknowns in the assay and reading them on the standard curve. In most cases, the specific activity was approximately 30 Ci/mM.

3. Antibody production

Nicergoline was coupled to bovine serum albumin in the presence of formaldehyde [7]. Coupling occurred at posi-

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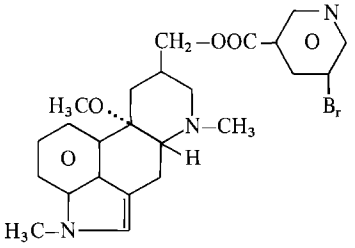

Fig. 1. Nicergoline

tion 1 of 1-demethyl nicergoline. Bovine serum albumin (210 mg, 3 μmol) was dissolved in 1 ml water and 2 ml 3 M sodium acetate at room temperature. Then 4 ml 37% formaldehyde solution was added dropwise and the mixture was stirred for 5 min. Then 25 mg 1-demethyl nicergoline dissolved in 1 ml water was added slowly. The resulting solution was stirred for 2 h at room temperature, then dialyzed versus distilled water at 4° C for 4 days. Analysis of the conjugate by UV spectrometry using the method described by Erlanger [4] indicated that there were 49 haptens bound to each protein molecule. The conjugate was lyophilized for storage.

The immunogen (0.5 mg) was supended in 1 ml physiological saline and emulsified with an equal volume of Freund's complete adjuvant. This suspension was injected ID into five rabbits using Vaitukaitis' method [9]. Booster injections of the same dose were given every 5 weeks. Samples were collected 10 days after each booster by puncturing the marginal ear vein. The antisera were evaluated (titre, affinity and specificity for the antibody) and, when the characteristics were satisfactory, a large volume of rabbit blood was obtained by cardiac puncture.

4. Assay procedure

The standard immunoassay curve was established and nicergoline levels in plasma and urine assayed by adding the reagents shown in Table 1 to 5-ml plastic tubes in the order indicated. Assays were conducted on volumes of plasma ranging from 10 to 100 μl, depending on the expected nicergoline plasma level. It was occasionally necessary to dilute a sample, in which case the nicergoline-free plasma sample had to be adjusted accordingly. In all cases, the reaction volume was kept to 500 μl. The same steps were taken for urine.

After subtracting the activity measured for tube D from that measured for tubes A, B and C, the standard curve was plotted and used to determine the nicergoline concentrations in the plasma and urine test samples.

Table 1. Operating protocol for radioimmunoassay of nicergoline

Tube		A	B	C	D	E
Buffer B1	μl	200	100	200	300	–
Nicergoline standard solution (30–2,000 ng/l)	μl	–	100	–	–	–
Test plasma or urine	μl	–	–	10–100	–	–
Nicergoline-free plasma or urine	μl	10–100	10–100	–	10–100	–
^{125}I-nicergoline solution (approx. 15,000 cpm)	μl	100	100	100	100	100
Antibody solution (1/5,000 dilution)	μl	100	100	100	–	–

The preparations were vortexed for 30 s. After incubating for 24 h at 4° C, the tubes were placed in an ice bath and 0.5 ml dextran-coated charcoal suspension was added; then they were vortexed. After 15 min, the tubes were centrifuged at 400 rpm, then decanted and the activity of the dextran-charcoal was determined. Each assay was performed in triplicate

Results

1. Anti-nicergoline antibodies

Of the seven immunized rabbits, three died shortly after immunization whereas, over a few months, the other four developed antibodies with quite similar characteristics. The antiserum dilution producing 40%–60% binding of the tracer was between 2,500 and 5,000. The antibodies obtained were studied for reaction with various molecules having chemical structures similar to that of nicergoline. The results are given in Table 2. Only nicergoline, 1-demethyl nicergoline and, to a lesser degree, 6-demethyl nicergoline reacted with the antiserum. All other molecules, particularly 1,6-demethyl-8-hydroxymethyl-10β-methoxy-α-ergoline which is apparently the primary metabolite of nicergoline in man and 5-bromonicotinic acid, caused little or no interference; the assay was therefore extremely specific for nicergoline. A study using the Scatchard procedure [8] on the antiserum that is currently in use revealed two high affinity antibody populations ($K_1 = 10^{10}\ l \cdot M^{-1}$, $K_2 = 1.5 \times 10^9\ l \cdot M^{-1}$).

Table 2. Specificity of Antiserum

Compounds	I%
Nicergoline	1
1-Demethylnicergoline	1
6-Demethylnicergoline	0.65
10-Methoxy-6-Methylnicergoline--8β-Carboxylic acid methylester	$<10^{-3}$
1,6-Dimethyl-8-hydroxymethyl--10β-methoxy α-Ergoline	$<10^{-3}$
Dihydroergotamine methanesulphonate	$<10^{-4}$
Methysergide maleate	$<1.5 \times 10^{-4}$
Dihydroergotoxine	$<2 \times 10^{-4}$
Dopamine	$<2 \times 10^{-6}$
Bromocriptine methanesulphonate	$<10^{-4}$

$$I\% = \frac{\text{mass of nicergoline required to displace 50\% of antibody-bound tracer}}{\text{mass of test compound required to displace 50\% of antibody-bound tracer}}$$

2. Storage of the tracer

Nicergoline labelled with ^{125}I keeps well for 30–40 days after labelling when it is maintained in alcohol at −20° C. During this time, binding to the antibody and reaction with the absorbant remain constant.

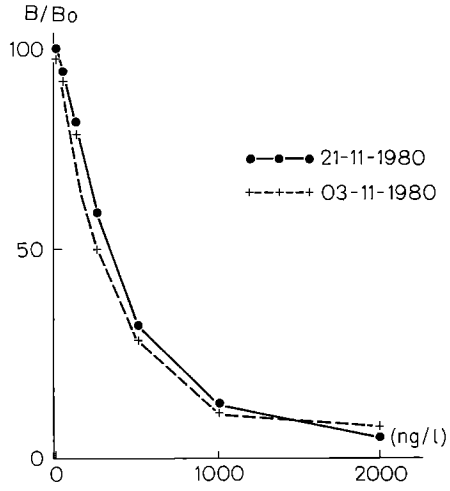

Fig. 2. Standard curves of nicergoline/Radioimmunoassay

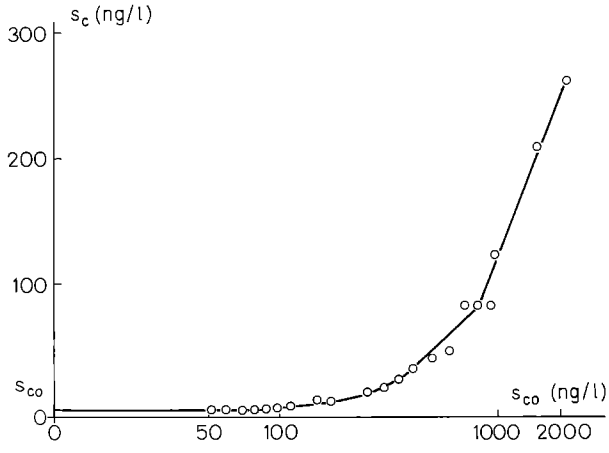

Fig. 3. Precision profile expressed in terms of absolute error as a function of concentration

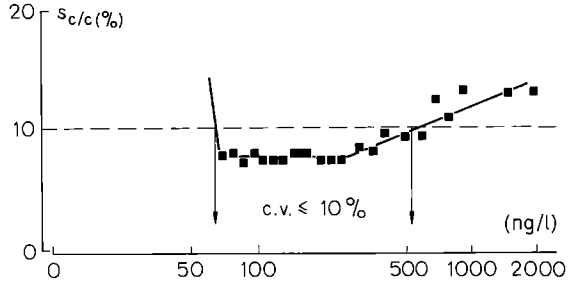

Fig. 4. Precision profile expressed in terms of relative error as a function of concentration

3. Standard curve

Standard curves were constructed from data obtained with standard preparations containing nicergoline-free plasma (10, 25 or 100 µl) as well as from data obtained with preparations containing no additional plasma. Addition of plasma to the reaction medium did not significantly modify the slope of the curve but resulted in a reduced percentage binding of tracer to antibody. For this reason, the standard series was prepared by adding to each standard solution a volume of plasma identical to that which was to assayed. Generally 10 µl plasma was used. In Fig. 2, two examples of standard curves obtained under these conditions using the same tracer and the same initial antibody dilution (1/2,500) are shown. These curves are very similar even though the assays were performed 3 weeks apart. In addition, the values for B/Bo were easily distinguished even at low concentrations.

4. Criteria for establishing the quality of the method. Intra-assay and inter-assay reproducibility. Precision profile. Detection limit

With a view to determining the intra-assay reproducibility of the method at all concentrations, i.e. to establish the precision profile, the operating procedure proposed by Ekins [3] was followed which also determines the detection limit for the method. During one study, ten assays were performed on each of a series of standard solutions containing 10 µl nicergoline-free plasma under the conditions described above. The mean response and standard deviation for each concentration and the slope of the curve were used to estimate the error s_c made in determining the concentration c. Figure 3 gives s_c as a function of c. The error made in measuring a zero concentration, S_{co}, indicates the smallest concentration of nicergoline that can be differentiated from zero, i.e. the method's detection limit. This was 6 ng/l under the conditions set down in the operating procedure. In plasma, the least detectable dose of the drug was dependent on the volume and the degree of plasma dilution. When 10 µl plasma was used, the most frequent case as already mentioned, the detection limit rose to 125×10^{-12} mol/l and reached 1.25×10^{-9} mol/l for an assay of 10 µl plasma diluted to 1:10.

Table 3. Results obtained after addition of nicergoline to human plasma initially nicergoline-free (Concentration expressed as ng/l)

Theoretical values	12	20	25	40	50	100
Values obtained during successive assays	12.5	21	27	34	49	93
			33		66	98
			31		61	92
Mean value			30		59	94
Percentage recovery	104	105	120	85	118	94
CV (%)			10		14.5	3.4

Fig. 4 shows s_c/c as a function of c, i.e. the intra-assay variation coefficient (%) was less than or equal to 10% in a wide concentration zone 60–600 ng/l) and did not exceed 14% for 2,000 ng/l. Both the sensitivity of the method and the intra-assay reproducibility were therefore good. This has been certified several times. Confirmation was obtained by establishing the precision profile from all the results for series including not only standard solutions but also plasma samples with unknown nicergoline concentrations as well as control plasma samples.

The precision of the method was studied by conducting several assays of nicergoline-free plasma samples (Po), nicergoline-free plasma samples to which the substance had been added, and samples rich in nicergoline which were diluted progressively with Po. The results are given in Table 3. The recovery was highly satisfactory (85%–120%). The inter-assay reproducibility was also satisfactory with a coefficient of variation of between 3.4% and 14.5%. The dilution test also gave acceptable results.

5. Pharmacokinetic studies

Having found a sensitive and specific assay method for nicergoline in the plasma, it was applied to a number of control subjects following administration of the drug. We used adult volunteers of both sexes, aged between 20 and 35 years. A large number of assays were performed and plasma concentrations under different conditions were determined with regard to dose and route of administration. The results will be the subject of an article to be published shortly.

Discussion

A method has been developed for assaying nicergoline in plasma and urine. To avoid the long and costly synthesis of tritium-labelled nicergoline, it was decided, on commencing our studies, to use an iodine-labelled tracer (^{125}I) that gives a much heavier molecule than the unlabelled ligand. This finally proved a valid choice as the method gives satisfactory results. The laboratory-produced antibodies reacted only with nicergoline and its demethyl derivatives. The principal metabolites did not interfere; this was also true for rye ergot derivatives. We are thus in possession of a specific method devoid of all interference which gives much more reliable results than those obtained by measuring the activity of biological samples following administration of tritium-labelled nicergoline to man or the animal.

In addition, the high specificity of the two antibody populations for the ligand produces high sensitivity. Assays can therefore be performed on extremely small samples of plasma or urine. In some cases, it is even necessary to dilute the sample before carrying out the determination. In such conditions, the body fluid has minimal influence on the antigen-antibody reaction. Nevertheless, it is preferable to prepare a standard series under the same conditions used for the biological samples, i.e. by adding the same volume of nicergoline-free body fluid to the standard series. The intra- and inter-assay variation coefficients and the results of recovery tests were satisfactory, therefore the method is relatively precise and reproducible, and the time needed to conduct the assay is quite short (24 h).

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