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Radioreceptor Assay for a New Antiallergic Agent, 1-(2-Ethoxyethyl)-2-(4-methyl-1-homopiperazinyl)benzimidazole Difumarate (KB-2413), in Plasma¹⁾

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A radioreceptor assay (RRA) for the quantitative determination of a new antiallergic agent, 1-(2-ethoxyethyl)-2-(4-methyl-1-homopiperazinyl)benzimidazole difumarate (KB-2413), has been developed. This RRA method, based upon competitive binding of [³H]mepyramine and KB-2413 to histamine H₁ receptors in guinea pig cerebellum, allows the simple, sensitive and reproducible determination of KB-2413 in plasma. The determination limit was 0.1 ng (as free base)/ml and the standard curve was linear over the range of 0.1 to 5.0 ng/ml. The intra- and inter-assay coefficients of variation for the determination of KB-2413 were 5.2—11.1% and 3.4—5.6%, respectively. The active metabolites, 1-(2-ethoxyethyl)-2-(1-homopiperazinyl)benzimidazole and 1-(2-ethoxyethyl)-5-hydroxy-2-(4-methyl-1-homopiperazinyl)benzimidazole, cross-reacted to the extents of 28.6 and 21.1%, respectively. However, plasma levels obtained by the RRA method and the gas chromatographic method for the intact drug were well correlated in guinea pigs after oral administration of KB-2413.

Keywords—antiallergic agent; 1-(2-ethoxyethyl)-2-(4-methyl-1-homopiperazinyl)benzimidazole difumarate; KB-2413; determination; radioreceptor assay; [³H]mepyramine; histamine H₁ receptor; plasma

Introduction

1-(2-Ethoxyethyl)-2-(4-methyl-1-homopiperazinyl)benzimidazole difumarate (KB-2413) has strong antiallergic activity and lower toxicity in animals as compared with other known antiallergic drugs such as ketotifen and chlorpheniramine.^{2,3)} The chemical structure of KB-2413 is shown in Fig. 1.

A method for determination of the intact drug in plasma was established by capillary gas chromatography (GC) with a nitrogen-sensitive detector and the usefulness of the method was confirmed in animal experiments at relatively high doses.⁴⁾ However, the dose in animal experiments was 50—100 times higher than the clinical dosage, so that a more sensitive determination method had to be developed to clarify the pharmacokinetics of KB-2413 in humans.

Radioreceptor assay (RRA) has proved to be valuable for measuring the plasma levels of a variety of drugs, for example, beta-blockers, benzodiazepines, neuroleptics, tricyclic antidepressants, calcium antagonists, *etc.* These RRAs are of great interest because of their

Fig. 1. Chemical Structure of KB-2413

ability to detect not only the parent drugs but also pharmacologically active metabolites by means of a simple technique.

Tran *et al.*⁵⁾ found that [³H]mepyramine selectively bound to histamine H₁ receptors in mammalian brain membranes and examined in detail the properties of histamine H₁ receptors in mammalian brain labeled with [³H]mepyramine.

We describe here an assay for a new antiallergic agent, KB-2413, based upon competitive binding of [³H]mepyramine and KB-2413 to histamine H₁ receptors in guinea pig cerebellum. The present assay is simple, sensitive, reproducible and suitable for routine clinical application.

Experimental

Materials—KB-2413 was synthesized and supplied by Fuji Chemical Industry, Co., Ltd. (Toyama, Japan). [³H]Mepyramine (specific activity; 26 Ci/mmol) used as a radioactive ligand was purchased from Amersham Japan Ltd. (Tokyo, Japan). 1-(2-Ethoxyethyl)-2-(1-homopiperazinyl)benzimidazole (metabolite A), 1-(2-hydroxyethyl)-2-(4-methyl-1-homopiperazinyl)benzimidazole (metabolite B), 1-(2-ethoxyethyl)-2-(4-methyl-4-oxide-1-homopiperazinyl)benzimidazole (metabolite C), 1-(2-ethoxyethyl)-5-hydroxy-2-(4-methyl-1-homopiperazinyl)benzimidazole (metabolite D), 1-(2-ethoxyethyl)-6-hydroxy-2-(4-methyl-1-homopiperazinyl)benzimidazole (metabolite E) and ketotifen fumarate were all synthesized at the Pharmaceuticals Research Center of Kanebo, Ltd. Antazoline phosphate, diphenhydramine hydrochloride, mepyramine maleate, methapyrilene fumarate, promethazine hydrochloride, triprolidine hydrochloride and tripeleminamine hydrochloride were obtained from Sigma Chemical Company (St. Louis, U.S.A.). Cyproheptadine hydrochloride was obtained from Nippon Merck Banyu Co., Ltd. (Tokyo, Japan) and clemastine fumarate from Sankyo Co., Ltd. (Tokyo, Japan). Benzene for pesticide analysis (used as an extraction solvent) and other reagents of special grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Membrane Preparations—Male Hartley strain guinea pigs (250–400 g, Keali Co., Ltd., Osaka, Japan) were decapitated. The cerebellums collected from more than 20 animals were homogenized in 30 volumes of ice-cold 50 mM Na/K phosphate buffer (pH 7.5) with a Physcotron (Niti-On Medical and Physical Instruments Mfg. Co., Ltd., Chiba, Japan) for 3 periods of 10 s at setting 55, and the homogenate obtained was centrifuged for 20 min at 25000 × *g* at 4 °C. The pellet was resuspended in the same volume of fresh phosphate buffer with a Potter-Elvehjem Teflon-glass homogenizer, and centrifuged in the same manner as above. The resulting pellet was stored at –70 °C until use, and homogenized in 60 volumes of ice-cold phosphate buffer with a Potter-Elvehjem Teflon-glass homogenizer when required for the receptor assay. Protein in membrane preparations was measured by the method of Lowry *et al.*⁶⁾

Receptor Assay—A 1 ml aliquot of plasma was mixed with 1 ml of 0.2 N sodium hydroxide and 6 ml of benzene in a 10-ml glass-stoppered centrifuge tube. The mixture was vigorously shaken for 10 min and centrifuged for 10 min at 3000 rpm. Then 5 ml of the organic layer was transferred into another 10-ml glass tube, and evaporated to dryness under a stream of nitrogen at about 40 °C. The residue was redissolved in 0.5 ml of 50 mM Na/K phosphate buffer (pH 7.5) and 0.2 ml of [³H]mepyramine solution in ice-cold phosphate buffer was added to give a final concentration of 0.9 nM. The mixture was pre-incubated for 1 min at 25 °C, then 0.3 ml of the membrane preparations containing approximately 0.34 mg of protein was added. Incubation was carried out at 25 °C for 30 min and was terminated by addition of 4 ml of ice-cold phosphate buffer. The mixture was immediately filtered onto a Whatman GF/C micro glass fiber filter under vacuum followed by washing three times with 4 ml of ice-cold phosphate buffer. A model VFM-1 cup-seal vacuum filtration manifold (Amicon Division, W. R. Grace & Co., Danvers, U.S.A.) and a model XX5500000 pump (Millipore Co., Bedford, U.S.A.) were used.

Radioactivity trapped on the filter was counted in 1 ml of methanol and 12 ml of Scintisol® EX-H (Dojindo Laboratories, Kumamoto, Japan) with a Tri-Carb liquid scintillation spectrometer (model B-2450, Packard Instrument Co., Downers Grove, Ill., U.S.A.). The counting efficiencies were determined automatically by the ²²⁶Ra external standard ratio method and cpm was converted to dpm.

The assay was always done in triplicate and standard curves were routinely obtained for each experiment by adding known amounts of authentic KB-2413 directly to blank plasma. Specific binding was calculated by subtracting nonspecific binding determined according to a similar procedure in the presence of 2 μM triprolidine from total binding.

The cross-reactivities of the main metabolites relative to KB-2413 were calculated from their affinity constants (*K_i* values) which were determined from the equation $K_i = IC_{50}/(1 + C/K_d)$, where *K_d* is the dissociation constant (0.27 nM) derived from the Scatchard analysis of specific [³H]mepyramine binding at 0.35 to 3.72 nM [³H]mepyramine and *C* is the concentration of the labelled ligand in the binding assay. The *IC*₅₀ values, the concentrations causing 50% inhibition of specific [³H]mepyramine binding, were calculated after linearization of binding inhibition data by

using a logit-log conversion.

GC Method—The capillary gas chromatographic method with a nitrogen-sensitive detector described in the previous report⁴⁾ was used.

Animal Experiment—Male Hartley guinea pigs (240–320 g, Keali Co., Ltd.) fasted overnight were used. An isotonic saline solution of KB-2413 (0.6 mg/ml) was administered orally at a dose of 2 mg/kg. At various times after dosing, the animals were anesthetized with ether, and blood samples were withdrawn from the abdominal vena cava with a heparinized plastic syringe. Plasma was promptly separated and stored at -20°C until analysis by the GC and RRA methods.

Results

Binding of [^3H]Mepyramine to Receptors

Total [^3H]mepyramine binding with the membranes of guinea pig cerebellum was approximately 5500 dpm (95 fmol) with nonspecific binding levels assayed in the presence of $2\ \mu\text{M}$ triprolidine being approximately 500 dpm (9 fmol). [^3H]Mepyramine binding was inhibited by the direct addition of plasma, for example, by 34.7 and 42.6% with 50 and 100 μl of plasma, respectively. On the other hand, only 5.8% of specific binding was inhibited by the benzene extracts of plasma, and the standard deviation of the inhibition was only 2.3% ($n=6$).

Stability of Brain Membranes

The pellet of brain membranes was stored for 3 months at 5, -20 and -70°C , and the variations of specific [^3H]mepyramine binding were examined. The brain membranes have

Fig. 2. Stability of Membrane Pellet on Storage at (■) 5°C , (▲) -20°C and (●) -70°C

Fig. 3. Standard Curve for the Determination of KB-2413 in Plasma

TABLE I. Intra-assay Analytical Precision for the Determination of KB-2413 in Plasma

KB-2413 (as free base) (ng/ml)		Coefficients of variation (%)	n
Added	Found		
0.1	0.10	10.0	5
0.25	0.26	8.1	5
0.5	0.48	5.2	5
1.0	0.99	6.0	5
2.5	2.53	11.1	5
5.0	5.11	9.3	5

TABLE II. Inter-assay Analytical Precision for the Determination of KB-2413 in Plasma

KB-2413 (as free base) (ng/ml)		Coefficients of variation (%)	n
Added	Found		
0.1	0.09	5.6	6
0.25	0.25	4.8	6
0.5	0.53	4.9	6
1.0	1.06	3.4	6
2.5	2.51	3.7	6

proved to be stable for at least 3 months at less than -20°C , as shown in Fig. 2. An extreme decrease of specific binding took place on storage at 5°C .

Standard Curve

Various amounts (0.1 to 5.0 ng) of KB-2413 were added to 1 ml aliquots of the blank plasma obtained from normal subjects. A standard displacement curve for KB-2413 was converted to a straight line by means of a logit-log plot. A typical example of the standard curves is presented in Fig. 3. It showed a good linearity and allowed us to determine KB-2413 at a concentration as low as 0.1 ng (as free base)/ml.

Accuracy and Reproducibility

The accuracy and reproducibility of the assay were evaluated at concentrations of 0.1 to 5.0 ng of KB-2413/ml. The intra- and inter-assay data are summarized in Tables I and II, respectively.

In the intra-assay study, the overall recoveries of KB-2413 from plasma samples averaged 101 ± 8.3 (S.D.) % and the coefficients of variation were 5.2–11.1% in the concentration range of 0.1 to 5.0 ng/ml. On the other hand, in the inter-assay study, the overall recoveries of KB-2413 from plasma samples averaged 99.9 ± 5.83 (S.D.) % and the coefficients of variation were 3.4–5.6% in the concentration range of 0.1 to 2.5 ng/ml.

Specificity

As shown in Table III, metabolites A and D possessed about 30 and 20% cross-reactivities for KB-2413, respectively, while the other metabolites were less than 1.7% cross-reactive.

Correlation of Affinity Constant and Pharmacological Activity

Affinity constants of various antihistaminic drugs including KB-2413 to $[^3\text{H}]$ mepyramine binding sites were determined from their IC_{50} values and compared with their potencies in blocking histamine-induced guinea pig ileal contraction (pA_2).^{2,7-9)} As shown in Fig. 4, there was a good correlation ($\gamma = 0.94$, $n = 10$) between them.

TABLE III. Cross-Reactivities of the Main Metabolites of KB-2413

Metabolites	Cross-reactivities (%)
A	28.6
B	0.8
C	0.6
D	21.1
E	1.7

A–E, see the text.

Fig. 4. Correlation of Affinities of Drugs for $[^3\text{H}]$ Mepyramine Binding Sites (pK_i) with Their Potencies in Blocking Histamine-Induced Guinea Pig Ileal Contraction (pA_2)

pA_2 values were derived from published data.^{2,7-9)} Slope = 0.82, $\gamma = 0.94$.

1, clemastine; 2, mepyramine; 3, KB-2413; 4, promethazine; 5, cyproheptadine; 6, ketotifen; 7, methapyrilene; 8, tripeleminamine; 9, diphenhydramine; 10, antazoline.

Fig. 5. Plasma Levels of KB-2413 Obtained by (▲) the GC Method and (●) RRA, and (■) Pharmacological Activity after Oral Administration of KB-2413 in Guinea Pigs

Plasma levels and pharmacological activities were obtained at doses of 2 and 0.00156–0.0125 mg/kg, respectively. Plasma levels are presented in terms of the free base of KB-2413.

Fig. 6. Comparison of the Results of Radioreceptor Assay and Gas Chromatographic Assay of KB-2413 in Plasma Obtained from Guinea Pigs after Oral Administration of KB-2413 at a Dose of 2 mg/kg

Plasma levels are presented in terms of the free base of KB-2413.

Comparison of the RRA and GC Methods

Guinea pigs were orally given KB-2413 at a dose of 2 mg/kg. Plasma levels determined by the RRA method as well as by the GC method for the intact drug are shown in Fig. 5, which also shows pharmacological activity, that is, the inhibitory effect on histamine-induced mortality of guinea pigs, at doses of 0.00156 to 0.0125 mg/kg.²⁾ Plasma levels of the intact drug reached the maximum (about 90 ng/ml) at 30 min after dosing and then decreased with a half life of about 1 h. Plasma levels measured by the RRA method were similar to but slightly higher than those by the GC method.

The correlation between the values measured by the two methods, based on a total of 21 samples, is shown in Fig. 6. There was a good correlation with a slope of 1.15 and a correlation coefficient of 0.99. In addition, the pharmacological activity reached the maximum at 1 h after dosing and then decreased with almost the same half-life as that of the plasma levels.

Discussion

Chang *et al.*¹⁰⁾ demonstrated that considerable species differences existed in the affinity of [³H]mepyramine to the brain membrane preparations of various animals. The affinities to guinea pig and human brain membrane preparations were 3–6 times greater than those to rat, mouse and rabbit preparations, and the greater affinity of [³H]mepyramine in guinea pigs was attributable both to faster association rates and slower dissociation rates in guinea pigs than in rats. Moreover, Chang *et al.*,¹⁰⁾ Tran *et al.*⁵⁾ and Hill *et al.*¹¹⁾ found pronounced differences between the regional localization of specific [³H]mepyramine binding in various animals.

In our preliminary studies, species differences and regional distribution were also observed in specific [³H]mepyramine binding to rat and guinea pig brains. Specific [³H]mepyramine binding to guinea pig cerebellum was about 3 times higher than that to guinea pig whole brain excluding cerebellum and about 7 times higher than that to rat whole brain. In the present study, therefore, we used guinea pig cerebellum as histamine H₁

receptors. The membrane pellet of guinea pig cerebellum was easily prepared and was stable for at least 3 months at less than -20°C (Fig. 2).

In the determination of KB-2413 in plasma by means of the RRA method, specific [^3H]mepyramine binding was inhibited by the direct addition of a small amount of plasma to the incubation mixture. Increasing volumes of plasma reduced the binding gradually, causing about 40% inhibition with 100 μl of plasma. This was presumably due to the binding of [^3H]mepyramine with plasma proteins. In order to decrease the inhibition of specific [^3H]mepyramine binding and raise the sensitivity of the determination of KB-2413 in plasma, the extraction of KB-2413 in plasma with benzene under basic conditions was carried out prior to the receptor assay. The inhibition of specific [^3H]mepyramine binding by benzene extracts was only 5.8%, and it had thus become feasible to determine 0.1 ng (as free base)/ml of KB-2413 in plasma by assaying the extracts of 1 ml of plasma (Fig. 3). Consequently, the sensitivity of the RRA method was about 20 times higher than that of the GC method,⁴⁾ and excellent reproducibility of the former method was indicated by the values in the intra- and inter-assay studies (Tables I and II).

The affinity constants (K_i value) of several antihistaminic drugs to histamine H_1 receptors correlated closely with their pharmacological activities, that is, potencies in blocking histamine-induced guinea pig ileal contraction (Fig. 4). It was suggested that KB-2413 was comparable in potency to the most active H_1 antihistaminics, ketotifen, cyproheptadine and mepyramine. Metabolites A (desmethylated compound) and D (5-hydroxylated compound) of the 5 main metabolites of KB-2413 possessed about 20–30% cross-reactivities for KB-2413 (Table III), so these metabolites might also have pharmacological activities.

The plasma levels of guinea pigs orally given 2 mg/kg of KB-2413 were determined by the RRA method and at the same time, the plasma levels of the intact drug were determined by the GC method. The levels obtained by the RRA method were similar to but slightly higher than those by the GC method (Figs. 5 and 6). It was considered that this slight difference was attributable to the presence of some active metabolites, for example, metabolites A and/or D. However, the substantial correspondence of the results of the RRA and GC methods suggested that presence of only small amounts of active metabolites in guinea pig plasma, if they are present. This will be examined in a future study.

The pharmacological activity (in terms of the inhibitory effect on histamine-induced mortality of guinea pigs) was compared with the concentration of KB-2413 in plasma after oral administration. The pharmacological activity reached the maximum about 30 min later than the peak of the plasma level, but their half-lives were comparable (Fig. 5). This time lag presumably represents the time required for KB-2413 to pass to the receptors from plasma, although the variations in experimental values need to be taken into consideration.

In conclusion, the present RRA method is simple, sensitive, reproducible and suitable for estimating the plasma levels, which well reflected the pharmacological activity. The usefulness of this RRA method in clinical studies was suggested in the preliminary report.¹²⁾ Further details will be reported elsewhere.

References and Notes

- 1) A part of this work was presented at the 105th Annual Meeting of the Pharmaceutical Society of Japan, Kanazawa, April 1985.
- 2) T. Fukuda, Y. Morimoto, R. Iemura, T. Kawashima, T. Tsukamoto and K. Ito, *Arzneim.-Forsch./Drug Res.*, **34**, 801 (1984).
- 3) T. Fukuda, T. Saito, S. Tajima, K. Shimohara and K. Ito, *Arzneim.-Forsch./Drug Res.*, **34**, 805 (1984); T. Fukuda, T. Saito and K. Ito, *ibid.*, **34**, 811 (1984); T. Fukuda, T. Saito, M. Yoshidomi and K. Ito, *ibid.*, **34**, 816 (1984).
- 4) T. Hamada, M. Kadowaki, Y. Nakamura and N. Awata, *Chem. Pharm. Bull.*, **34**, 1168 (1986).
- 5) V. T. Tran, R. S. L. Chang and S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 6290 (1978).

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- 6) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
 - 7) J. D. P. Graham, *J. Pharmacol. Exp. Ther.*, **91**, 103 (1947).
 - 8) P. B. Marshall, *Br. J. Pharmacol. Chemother.*, **10**, 270 (1955).
 - 9) G. V. R. Born, O. Eichler, A. Farah, H. Herken and A. D. Welch, *Handbook of Experimental Pharmacology*, **XVIII**, 1978.
 - 10) R. S. L. Chang, V. T. Tran and S. H. Snyder, *J. Neurochem.*, **32**, 1653 (1979).
 - 11) S. J. Hill, P. C. Emson and J. M. Young, *J. Neurochem.*, **31**, 997 (1978).
 - 12) T. Hamada, M. Kadowaki, Y. Nakamura and N. Awata, The 106th Annual Meeting of the Pharmaceutical Society of Japan, Chiba, April 1986.