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# Radioimmunoassay for Clebopride, a New Benzamide Drug with Antidopaminergic Activity

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A radioimmunoassay method has been developed for the measurement of low concentrations of clebopride in human serum and urine samples. Antisera (clebopride-binding antibodies) were obtained from albino rabbits eight weeks after the first injection of clebopride–bovine serum albumin (BSA) conjugates. The antisera, $^3$H-antigen and human serum (or urine) samples or standard clebopride solutions were used in the incubation mixtures for the assay. The method has been found to be very specific, because although clebopride is extensively metabolized in man, only clebopride and its glucuronide conjugate were found to be susceptible to the antisera. The unchanged drug could be separated from the conjugate by ether-extraction prior to assay. The limit of sensitivity of this method is 0.1 ng of clebopride per ml of sample and is sufficient to measure clebopride concentrations in serum and urine of human subjects after oral administration of the therapeutic dose of 0.5 mg per man. Serum concentration profiles and some of the pharmacokinetic properties of clebopride in man have been elucidated for the first time.

**Keywords**——radioimmunoassay; clebopride; benzamide; antidopaminergic activity; serum level; urinary excretion; specific antibody

Clebopride malate,$^{1)}$ 4-amino-*N*-(1-benzyl-4-piperidyl)-5-chloro-*o*-anisamide malate, is a new benzamide drug developed by Laboratorios Almirall, Spain, and it has potent central antidopaminergic and antiemetic properties.$^{1-8)}$ The drug increases the rate of gastric evacuation and is used for patients with gastrointestinal ulcers.$^{1-3,9-11)}$ Clebopride is extensively metabolized in different species, including man;$^{12,13)}$ however, the therapeutic doses are comparatively low, as low as 0.5 mg/man, resulting a very low concentration of clebopride in the plasma. Several analytical methods$^{14-16)}$ have been used to measure the plasma concentration of this drug by using thin-layer chromatography (TLC) or gas chromatography-mass spectrometry (GC-MS) but none of these methods are suitable for the determination of human plasma levels of clebopride due to the limit of sensitivities of the methods. Radioimmunoassay (RIA) is one of the most sensitive and widely used methods to determine the blood concentrations of many drugs.$^{17,18)}$ This led us to establish a new, highly sensitive and specific RIA method to measure clebopride concentrations in blood and other body fluids. In this paper we describe the details of our RIA method together with some applications to human serum and urine to measure clebopride levels after oral administration of a therapeutic dose.

## Materials and Methods

Clebopride malate, 4-amino-5-chloro-*N*-(4-piperidyl)-*o*-anisamide (desbenzyl clebopride), and 4-amino-*N*-(1-benzyl-4-piperidyl)-5-chlorosalicylamide (desmethyl clebopride) were the products of Laboratorios Almirall, Spain. Clebopride $N^4$-glucosiduronic acid (CP-G) was prepared by the method of Arita *et al.*,$^{19)}$ with a purity of 91.5% as determined by C, H, N analysis.

**Preparation of Immunogen, Clebopride–Bovine Serum Albumin (BSA) Conjugates**——(1) Synthesis of $N^4$-Succinylclebopride Hemihydrate: One hundred mg (0.27 mmol) of clebopride and 40 mg (0.4 mmol) of succinic anhydride were treated with 1 ml of acetic acid in an oil-bath at 90 °C for 3.5 h. Then the solvent was evaporated off and the oily residue was recrystallized from methanol–ethyl acetate. The crystalline colorless product (100 mg; yield, 78%) had a melting point of 169—170 °C. (2) Synthesis of Clebopride–BSA Conjugates: A pyridinic solution of $N^4$-succinylclebopride hemihydrate (100 mg/2 ml) was added dropwise to an aqueous solution of 100 mg of BSA. Then 38.4 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was added to the solution; the reaction was completed by overnight stirring in the dark and the solution was dialyzed against 3 l of 0.05 M potassium phosphate buffer (KPB) containing 0.15 M NaCl (pH 7.2), at 4 °C for 2 d with several changes of the buffer. The dialyzed solution containing 12.35 mg/ml of clebopride–BSA conjugates was sterilized by passage through a microfilter (pore size, 0.22 $\mu$m; Fuji Photo Film Co., Ltd., Tokyo) and kept frozen until use. In this preparation, about thirteen to sixteen molecules of clebopride were coupled to a BSA molecule as determined by ultraviolet (UV) analysis.

**Synthesis of $^3$H-Labeled Clebopride**——A solution of 5 mCi of dimethyl sulfate (methyl-$^3$H, 2.1 Ci/mmol; New England Nuc. Corp., Boston, Mass.) in 100 $\mu$l of tetrahydrofuran was added to 3.6 mg desmethyl clebopride Na-salt and the mixture was heated at 50 °C for 3 h under constant stirring. The product was purified by extraction with chloroform at alkaline pH, followed by washing of the organic layer with distilled water. The final preparation, containing 1.24 mCi of $^3$H-clebopride (Fig. 1) with 49.6% recovery and 1.05 Ci/mmol specific activity, gave a single radioactive spot at *Rf* 0.59 on TLC using a solvent system of *n*-BuOH–AcOH–$H_2O$ (4 : 1 : 1; v/v).

**Preparation of $^3$H-Antigen, Clebopride Hemisuccinyl-$^3$H-Tyrosine Methyl Ester**——A solution of L-(2,3,5,6-$^3$H)-tyrosine, 1 mCi (89 Ci/mmol), in 0.3 ml of 10% HCl–methanol was allowed to stand for 2 h at room temperature, then evaporated. The residue was transferred into a micro test tube. An aliquot (60 $\mu$l) of a tetrahydrofuran solution (20 ml) containing 10 mg of $N^4$-succinylclebopride, 10 mg of *N*-hydroxybenzotriazole and 25 $\mu$l of *N*-methylmorpholine was added to the micro test tube, followed by the addition of an aliquot (30 $\mu$l) of a tetrahydrofuran solution (10 ml) containing 10 mg of dicyclohexylcarbodiimide. The solution was stirred overnight and then the solvent was removed. The residual products were separated on a silica gel—thin layer plate using chloroform–methanol (13 : 1) solvent. The desired product (*Rf* 0.25) was eluted with chloroform–methanol (1 : 1) and stored at −20 °C in an ethanolic solution. The yield was 20% (200 $\mu$Ci) and the product showed nearly 100% radiochemical purity. The structures of clebopride and its derivatives are shown in Fig. 1.

**Immunization**——Clebopride–BSA conjugate (2 mg) emulsified with complete Freund's adjuvant was injected intradermally at multiple sites of the foot-pads of four adult albino rabbits. Booster injections with 1 mg of the emulsified immunogen were given intradermally in the abdomen and intramuscularly in the hip every two weeks. Eight weeks after the initial immunization, sera were obtained from the rabbits and stored at −20 °C until use.

Antiserum with the highest titer, which bound with 45% of the $^3$H-antigen at a final dilution of 1 : 125000, was used in the following experiments.

**Radioimmunoassay**——The assays, all in duplicate, were performed in serological test tubes. The procedures to assay clebopride from human serum or urine and to obtain a six-point standard curve for clebopride were as follows.

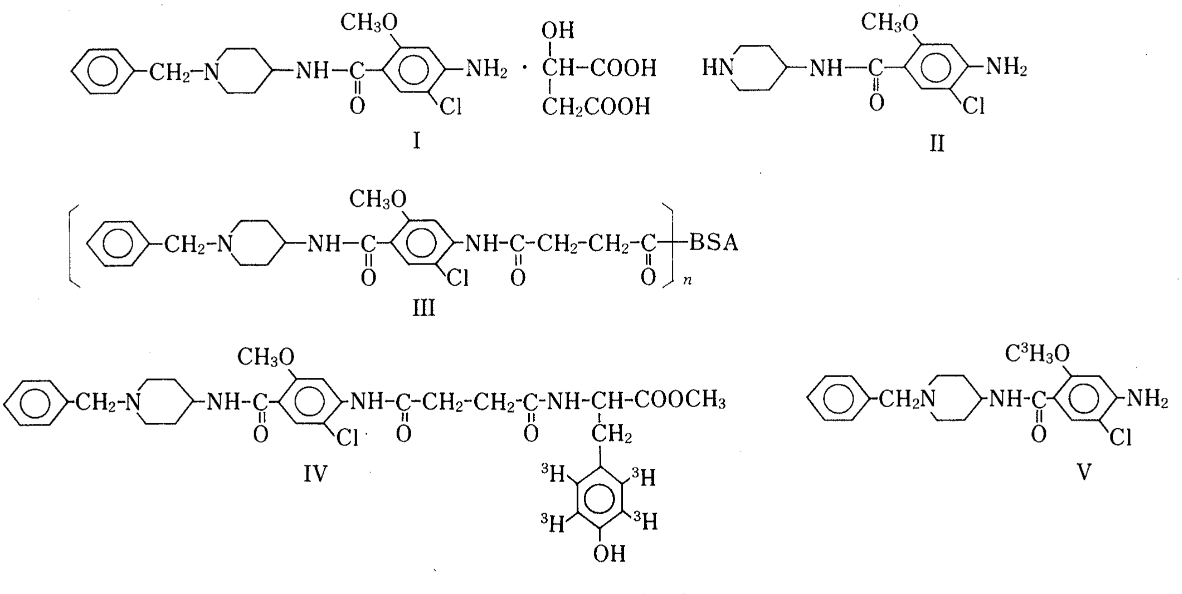

Fig. 1. Structures of Clebopride and Its Derivatives

I, clebopride malate; II, desbenzyl clebopride; III, clebopride hemisuccinyl BSA; IV, clebopride hemisuccinyl-$^3$H-tyrosine methyl ester ($^3$H-hapten) and V, $^3$H-clebopride.

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TABLE I. Effect of CP-G on Ether Extraction of Clebopride from Serum

| Ether extraction | CP-G added (ng/ml)[a] | Concentration of clebopride (ng/ml)[b] |
|---|---|---|
| Without | 0 | $2.16 \pm 0.16$ |
| With | 0 | $2.21 \pm 0.21$ |
| With | 1 | $2.20 \pm 0.12$ |
| With | 4 | $2.38 \pm 0.13$ |
| With | 16 | $2.29 \pm 0.21$ |

*a*) CP-G test solutions of constant clebopride concentration and various CP-G concentrations (16, 4 and 1 ng/ml) were prepared by using an aqueous solution of clebopride malate (1 mg clebopride/ml) diluted appropriately with control human serum, and the ether-extracted clebopride was assayed.

*b*) Mean ± S.D. ($n=5$).

A stock solution of clebopride malate (concentration of clebopride, 1 mg per ml) was diluted with control human serum or urine to give standard solutions of 10.0, 4.00, 1.60, 0.64, 0.26 and 0.10 ng of clebopride per ml. One hundred microliters of serum (or urine) samples or standard solutions, 0.3 ml of $^3$H-antigen diluted with KPB–0.5% BSA (approx. 10000 cpm), and 0.1 ml of diluted antiserum (1 : 25000) were added successively into the tubes. All tubes were vortexed and left at room temperature for 20 min. When the unchanged drug and glucuronide conjugate were separately measured, serum (or urine) samples or standard solutions were extracted with ether before mixing with $^3$H-antigen and antiserum. (The details of the separation procedure will be given under Separation and Determination of Clebopride and CP-G.)

In order to separate free $^3$H-antigen from bound form, 0.1 ml of 1% bovine γ-globulin (Sigma Chem. Co., St. Louis, MO) and 2 ml of 22% polyethyleneglycol diluted with KPB were added; the mixture was vortexed and centrifuged at 1600 × *g* for 20 min. The supernatant was aspirated; the precipitate was dissolved in 1 ml of 0.1 N NaOH and transferred into a vial containing 10 ml of toluene-Triton scintillation cocktail. The radioactivity was measured in a Packard Tri-Carb liquid scintillation counter with an efficiency of 27—28% for tritium.

**Collection of Human Serum and Urine Samples**——After a 16-h fast, five healthy adult male volunteers (average age, 29 yr; average weight, 65 kg) were given orally a clebopride malate tablet containing 0.5 mg of clebopride, with 100 ml of water. Blood samples of approximately 5 ml were collected from an elbow vein before, and 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h after the administration of the drug. The sera were separated from the clotted blood by centrifugation and stored at −20 °C until use. Urine samples were collected before and at intervals of 0—2, 2—4, 4—6, 6—8, 8—12 and 12—24 h after the dose and preserved at −20 °C with sodium axide (final concentration, 0.02%).

**Separation and Determination of Clebopride and CP-G**——A serum or urine sample (100 μl), mixed with 0.1 ml of 0.1 M borate buffer (pH 9.5), was added to 1 ml of ethyl ether. The mixtures were vortexed and centrifuged at 1600 × *g* for 5 min, then the organic layer was collected. The process was repeated twice and the combined ether phase containing clebopride was dried and subjected to RIA. The efficiency of the extraction process was monitored by estimation of the radioactivity in the organic layer by using $^3$H-clebopride, and it was found to be $89.5 \pm 1.1\%$ and $90.8 \pm 3.3\%$ for serum and urine, respectively. It was also observed that the co-existence of CP-G (0—7 times more than clebopride) in serum barely affected the efficiency of extraction of clebopride, as shown in Table I. The standard solutions were also treated in the same manner.

In the case of urine samples, the aqueous phase containing CP-G after ether extraction was acidified with 0.2 ml of 2 N HCl and then hydrolyzed at 70 °C for 1 h. The extent of hydrolysis monitored by using CP-G dissolved in human urine was $96.5 \pm 1.6\%$. After the hydrolysis of CP-G, the pH of the solution was adjusted to 9.5 by the addition of 2 N NaOH and the product, clebopride, was extracted with ether and assayed.

**Tests for Time and Temperature Dependency**——The mixtures of $^3$H-clebopride or $^3$H-antigen and anti-clebopride rabbit antiserum were incubated at 4 or 25 or 37 °C at various time intervals (0 to 24 h). After the incubation, γ-globulin and polyethyleneglycol were added, and the mixtures were centrifuged to collect the precipitates containing $^3$H-clebopride bound antibody. The radioactivities of the precipitates were measured.

## Results and Discussion

Huizing *et al.* established a rapid thin-layer chromatographic densitometric method for the determination of clebopride in biological fluids.[14] However, the method was only suitable to measure plasma level following doses of more than 10 mg/kg body wt in the experimental

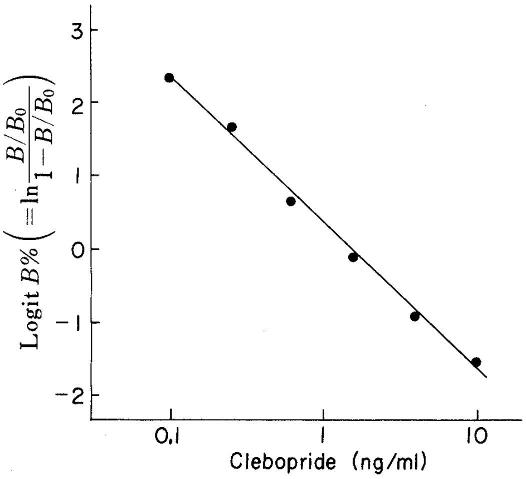

Fig. 2. Calibration Curve of Clebopride

Procedures were the same as described under Materials and Methods. The abscissa represents the logarithmic clebopride concentration *vs.* the logit B% [$=\ln(B/B_0)/(1-B/B_0)$] on the ordinate, where $B$ and $B_0$ represent the radioactivities (cpm or dpm) of bound $^3$H-hapten in the presence and absence of unlabeled clebopride, respectively. Each value was obtained by subtracting the blank value of the radioactivity without antiserum.

animals. Recently Hayasaka *et al.* reported a mass fragmentographic method for the measurement of unchanged clebopride and its main metabolites (total clebopride) in rat whole blood,[16)] but a more sensitive method is necessary to determine picogram levels of clebopride in the blood of human beings receiving therapeutic doses in clinical studies. We have established the present radioimmunoassay method for the determination of clebopride in the blood of human subjects after administration of the therapeutic dose.

Incubation time to complete the binding of antigen and antibody is one of the important factors in immunoassay. Our experiments on the time dependency show that the antigen–antibody complex formation was completed immediately after the mixing of $^3$H-clebopride or $^3$H-antigen and anti-clebopride rabbit antiserum. It was also found that the incubation at various temperatures (4, 25 and 37 °C) had no effect on the complex formation.

The method has been proved to be highly sensitive. The calibration curve of clebopride was obtained by using standard clebopride solutions diluted appropriately with control human serum. As shown in Fig. 2, the calibration plot was linear in the range of 0.1 to 10 ng/ml, and the minimum detectable concentration of clebopride was about 0.1 ng/ml (equivalent to 0.01 ng of clebopride in an assay tube). Similar results were obtained when control human urine instead of serum was used for standard solutions.

### Separation of Clebopride from CP-G

Clebopride is extensively metabolized in man and other animals.[12)] However, our understanding of clebopride metabolism in man is incomplete because of the low dosage used and the extensive metabolism of the drug, resulting in very low concentrations of the metabolites in the samples to be analyzed. The latest report[13)] shows that the major metabolites in man are desbenzyl clebopride, CP-G and $N^4$-glucosiduronic acid conjugate of desbenzyl clebopride (desbenzyl CP-G). It is very important for the RIA method to determine whether or not these major metabolic products of clebopride bind to the anti-clebopride antiserum used in the assay. For this purpose, inhibitory effects of clebopride and metabolites against $^3$H-antigen binding to the antibodies were tested. Relative binding activities of CP-G and desbenzyl CP compared to that of clebopride (taken as 100%) were 57.5% and less than 0.02%, respectively. From this result, it can be said that the antibodies produced by animals immunized with the immunogen, clebopride–BSA conjugates, recognize the benzyl moiety of the clebopride molecule. This is reasonable because the opposite site to the benzyl moiety of the drug participates in linkage of an amino group of BSA *via* the succinyl bridge of the conjugate antigen.

To avoid the interference of CP-G in the measurement of unchanged clebopride, the ether-extraction technique was very useful; the recovery of clebopride was about 90%, while

TABLE II. Coefficients of Variation in the RIA Method

| | Concentration of clebopride (ng/ml)[a] | C.V. (%) | Average C.V. (%) |
|---|---|---|---|
| Intra-assay | $0.42 \pm 0.03$ | 5.9 | |
| | $1.34 \pm 0.08$ | 6.3 | 6.5 |
| | $4.35 \pm 0.31$ | 7.2 | |
| Inter-assay | $0.38 \pm 0.05$ | 14.1 | |
| | $1.29 \pm 0.10$ | 7.7 | 9.9 |
| | $4.42 \pm 0.35$ | 7.9 | |

[a] Sera (0.1 ml aliquot) from volunteers given clebopride malate orally were analyzed by RIA with ether extraction. Standard samples were also treated in the same way. Data represent means ± S.D. ($n=9$).

that of CP-G was negligible.

**Reproducibility of the Assay**

Three serum samples taken from volunteers who had been given clebopride malate orally were used for precision studies on between-assay (intra-assay) and between-day (inter-assay) variations. All the assays were carried out by the same workers. Coefficients of intra-assay and inter-assay variation (C.V.) were 6.5 and 9.9%, respectively, indicating that the limit of variation is acceptable, as shown in Table II.

**Application of the Method**

Serum samples from 5 male volunteers orally given 0.5 mg of clebopride (approx. 7.7 $\mu$g/kg body wt.) were analyzed by our RIA method. Serum levels of the drug were determined without and with ether extraction of free clebopride. In Fig. 3, the difference between two curves, without and with extraction, shows the contribution of CP-G (which is cross-reactive with the antiserum used in the assay). The calculation of CP-G might be possible by using Eq. 1; where $G$, $U$ and $M$ represent serum levels of CP-G, clebopride and

$$G=(\mathrm{M}-U)/0.575 \qquad (1)$$

clebopride plus CP-G, respectively; $U$ and $M$ are the measured values with and without ether extraction, respectively, and 0.575 is the relative binding activity of CP-G to the antibodies. An estimated CP-G curve is also shown in Fig. 3. All values of $M$, $U$ and $G$ are expressed as equivalent to clebopride.

Unlike urine, serum clots easily at 70 °C, resulting in low and variable recovery of clebopride as a hydrolysis product of CP-G. To overcome this difficulty, the following techniques might be used to measure serum CP-G; one is to hydrolyze CP-G with glucuronidase prior to RIA; another is to prepare a calibration curve with synthetic CP-G for direct measurement of CP-G after the removal of clebopride.

The urinary concentrations of clebopride and CP-G were each successfully measured as described in Materials and Methods. The cumulative excretion values are shown in Fig. 4. During 24 h after oral administration of 0.5 mg of clebopride, only 1.1% of clebopride and 1.4% of CP-G were excreted in the urine. Segura *et al.*[12] administered an oral dose of clebopride malate equivalent to 10 mg of clebopride base and the urine was collected up to 6 h after the dose; however, they mentioned that they could not detect $N^4$-glucuronide conjugate in the urine of man. Our present RIA method for clebopride was sensitive enough to measure such low concentrations of clebopride as are present in the urine of man after a therapeutic dose of the drug.

There has been a report[15] on the pharmacokinetics of clebopride in the rat and the dog

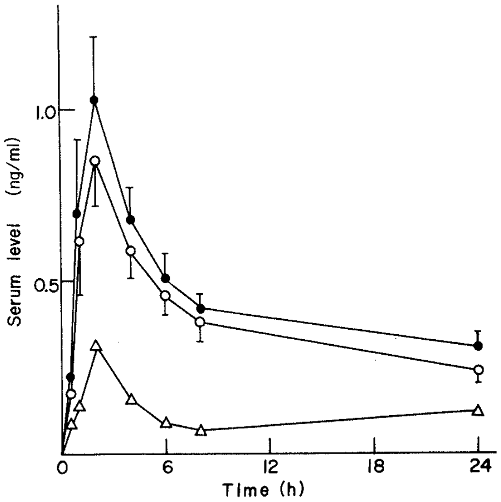

Fig. 3. Serum Concentrations of Clebopride and CP-G in Man

Five healthy volunteers were given orally a clebopride malate tablet containing 0.5 mg of clebopride and then the serum samples were assayed as described under Materials and Methods.

●: Mixture of clebopride and CP-G (without ether extraction).

○: Unchanged clebopride (with ether extraction).

△: CP-G concentration.

All concentrations are expressed as equivalent to clebopride.

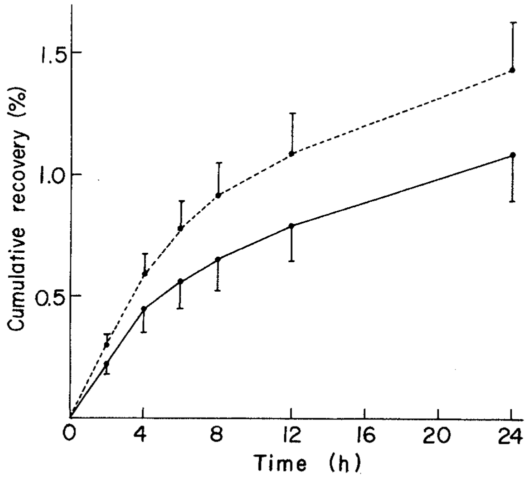

Fig. 4. Urinary Excretion of Clebopride and CP-G

Urine samples were collected from the same volunteers as mentioned in Fig. 3, as described under Materials and Methods. Cumulative recoveries (%) of clebopride and CP-G are shown by the solid line and the broken line, respectively.

TABLE III. Pharmacokinetic Parameters of Clebopride in Man

| Parameters | *U* | *M* |
|---|---|---|
| $C_{max}$ (ng/ml) | 0.87 | 1.05 |
| $T_{max}$ (h) | 1.61 | 1.63 |
| *AUC* (ng·h/ml) | 17.01 | 26.70 |
| $T_{1/2\alpha}$ (h) | 1.33 | 1.24 |
| $T_{1/2\beta}$ (h) | 23.90 | 36.47 |
| $K_{el}$ ($h^{-1}$) | 0.094 | 0.089 |

*U*, free clebopride concentration (with ether extraction); *M*, clebopride plus CP-G concentration (without ether extraction); $C_{max}$, maximum serum level; $T_{max}$, time at maximum level; *AUC*, area under curve of the serum level *vs* time; $T_{1/2\alpha}$, half-life in $\alpha$-phase; $T_{1/2\beta}$, half-life in $\beta$-phase; and $K_{el}$, elimination rate constant.

Parameters were calculated by the method of residuals fitted to a two-compartment open model.

but in that report it was said that the then-available methods of analysis were not sufficiently sensitive to measure clebopride in man because of the high volume of distribution of the drug and the low clinical dose. As shown in Table III, some pharmacokinetic parameters of clebopride in man have now been derived from the data presented in Fig. 3. This is the first time that the pharmacokinetic properties of clebopride have been measured in man, and further details will be published elsewhere. Our new, sensitive method for the assay of clebopride should be most useful in studies leading to a better understanding of the clinical and pharmacological properties of this new drug.

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