Dosage Form Design for Improvement of Bioavailability of Levodopa III: Influence of Dose on Pharmacokinetic Behavior of Levodopa in Dogs and Parkinsonian Patients

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Abstract □ The relationship between the dose of levodopa and its pharmacokinetic behavior following intravenous and oral administration was investigated in dogs and parkinsonian patients. Six beagle dogs received single doses of 2.4, 4.8, and 9.6 mg of levodopa/kg iv and single doses of 4.8, 9.6, and 19.2 mg of levodopa/kg po in a crossover fashion on separate occasions. Three parkinsonian patients received single oral doses of ~3.8, 7.7, and 15.4 mg of levodopa/kg in a crossover test. Plasma samples were analyzed for intact levodopa and total dopamine. The relationship between the area under the plasma concentration–time curve (*AUC*) of levodopa and the intravenous dose to dogs was linear. However, in both dogs and patients, the relationship after oral dosing was nonlinear, with the relative *AUC* increasing with increasing dose. Therefore, the pharmacokinetic behavior of levodopa after oral administration to dogs and patients was dose dependent.

Keyphrases □ Levodopa—pharmacokinetics, effect of dose, dogs and parkinsonian patients □ Pharmacokinetics—levodopa, effect of dose, dogs and parkinsonian patients □ Bioavailability—levodopa, effect of dose, dogs and parkinsonian patients

It was shown previously (1, 2) that the bioavailability of intact levodopa after oral administration was ~35% in both dogs and parkinsonian patients but that the total amount absorbed, including levodopa metabolites, was 80–90% of the administered dose, probably due to extensive metabolism of levodopa during absorption (2). On the other hand, Cotzias *et al.* (3) succeeded in treating parkinsonian patients with high doses of levodopa, and other investigators reported the effectiveness of high doses and the ineffectiveness of low doses in levodopa therapy (4–7).

The first-pass metabolism and clinical facts described indicate the need for an investigation of the absolute bioavailability of levodopa at different doses after intravenous and oral administration in beagle dogs and in patients.

EXPERIMENTAL

Intravenous Dose Studies of Levodopa in Dogs—Six healthy male beagle dogs, 10.3–13.3 kg, were fasted for ~16 hr and divided into three groups of two dogs each. Levodopa injection solution[1] was injected intravenously, over 30 sec, at 2.4 mg/kg to the first group, at 4.8 mg/kg to the second group, and at 9.6 mg/kg to the third group. Crossover experiments were carried out at 1-week intervals.

Blood samples were withdrawn with a heparinized syringe from the contralateral brachial vein. Sampling times were 0, 2, 5, 15, and 30 min and 1, 1.5, 2, 3, 4, and 6 hr postdosing. Urine was collected before the dose and over 48 hr after intravenous administration. The blood and urine specimens were processed as described previously (1, 2).

Oral Dose Studies of Levodopa in Dogs—The same six beagle dogs that were used in the intravenous dose studies were fasted for ~16 hr and divided into three groups of two dogs each. Levodopa in capsule form[2] was administered forcefully orally at 4.8 mg/kg to the first group, at 9.6 mg/kg to the second group, and at 19.2 mg/kg to the third group with 10 ml of warm water. Emesis was prevented by keeping the mouth closed by hand. Crossover experiments were carried out at 1-week intervals.

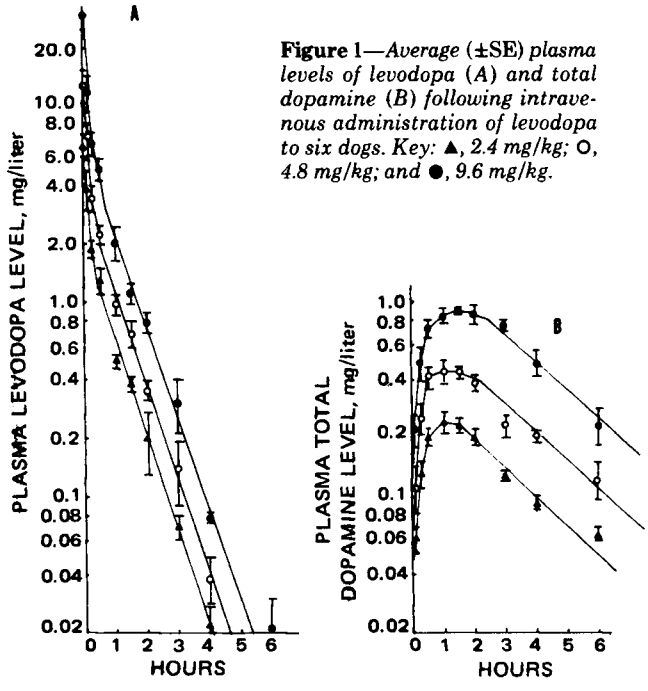

Figure 1—*Average (±SE) plasma levels of levodopa (A) and total dopamine (B) following intravenous administration of levodopa to six dogs. Key: ▲, 2.4 mg/kg; ○, 4.8 mg/kg; and ●, 9.6 mg/kg.*

Blood samples were withdrawn with a heparinized syringe before and at 0.5, 1, 2, 3, 4, and 6 hr after oral administration. Urine also was collected before the dose and over 48 hr. The blood and urine samples were processed as described previously (1, 2).

Oral Dose Studies of Levodopa in Parkinsonian Patients—Three parkinsonian patients, 26–63 years old, participated in this study. Three days prior to the experiments, all levodopa preparations and other drugs administered for the treatment of Parkinson's disease were withdrawn. Levodopa capsules[3] were administered orally at ~3.8 mg/kg together with a cup of water at ~9 am to the first subject, at ~7.7 mg/kg to the second subject, and at ~15.4 mg/kg to the third subject in the same manner. Crossover experiments were carried out at 1-week intervals.

On the day of the experiment, the patients ate breakfast at ~7:00 am, lunch at about noon, and dinner at ~5:30 pm. Blood was withdrawn with a heparinized syringe before dosing and at 0.5, 1, 2, 3, 4, and 6 hr after oral administration. The blood sample processing was carried out as described previously (1, 2). During the 3 days between the experiments of the crossover test, to prevent the appearance of parkinsonian syndrome, 500-mg doses of levodopa capsules were administered three times per day ~30 min after each meal instead of withdrawing the drug.

Assay of Levodopa and Its Metabolites in Plasma and Urine—Levodopa and its metabolites in plasma and urine were assayed according to the method reported previously (1).

[1] Dopaston injection, Sankyo Co., Ltd., Tokyo, Japan.

[2] Levodopa in capsule form was prepared in the same prescription preparation as Dopaston capsules, Sankyo Co., Ltd., Tokyo, Japan.

[3] Dopaston capsules, Sankyo Co., Ltd., Tokyo, Japan.

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Table I—Average (±*SE*) Pharmacokinetic Parameters of Levodopa and *AUC* of Levodopa and Total Dopamine following Administration of 2.4, 4.8, and 9.6 mg of Levodopa/kg to Six Dogs

Parameter	Dose, mg/kg		
	2.4	4.8	9.6
α, hr^{-1}	13.7 ± 2.8	13.6 ± 3.5	14.4 ± 3.0
β, hr^{-1}	1.16 ± 0.10	1.08 ± 0.12	1.04 ± 0.11
$0.693/\beta$, hr	0.62 ± 0.04	0.65 ± 0.05	0.67 ± 0.06
k_{12}, hr^{-1}	6.7 ± 0.61	7.1 ± 0.58	6.8 ± 0.57
k_{21}, hr^{-1}	3.5 ± 0.31	3.4 ± 0.39	3.7 ± 0.31
k_{10}, hr^{-1}	4.5 ± 0.41	4.4 ± 0.34	4.7 ± 0.38
V_1, liter/kg	0.25 ± 0.025	0.23 ± 0.021	0.27 ± 0.024
AUC of levodopa, (mg hr)/liter/kg	0.198 ± 0.015	0.359 ± 0.031	0.751 ± 0.069
Clearance of levodopa, liters/kg/hr	1.14 ± 0.11	1.18 ± 0.13	1.10 ± 0.12
AUC of total dopamine, (mg hr)/liter/kg	0.106 ± 0.009	0.215 ± 0.017	0.418 ± 0.035
Ratio of *AUC* for total dopamine to levodopa	0.52 ± 0.04	0.58 ± 0.04	0.56 ± 0.05

Pharmacokinetic Analysis—The plasma levodopa concentration curves following intravenous administration to dogs were analyzed using the program of Hanano *et al.* (8). The plasma levodopa concentration curves following oral administration to dogs and patients were analyzed using the BMDP-3R nonlinear regression program (9) with a one-compartment open model.

RESULTS

Intravenous Dose Studies of Levodopa in Dogs—The average plasma levels of levodopa and total dopamine[4] following intravenous administration of 2.4-, 4.8-, and 9.6-mg/kg doses of levodopa to six dogs are shown in Fig. 1. Plasma levels of levodopa and total dopamine increased proportionally with the dose of levodopa administered. The linear relationship between the dose and the *AUC* of levodopa is shown in Fig. 2. Pharmacokinetic parameters of the disposition of intravenously administered levodopa were estimated for each dog by fitting the data to the two-compartment open model, assuming linear kinetics. The resultant parameter estimates are summarized in Table I. The *AUC* values of levodopa and total dopamine corrected for body weight also are shown in Table I, as is the ratio of the *AUC* values of total dopamine to levodopa. This ratio, as well as the parameter estimates, is essentially constant and independent of the dose of levodopa administered.

The average urinary excretion of levodopa and its metabolites over 48 hr after intravenous administration of 2.4, 4.8, and 9.6 mg of levodopa/kg to six dogs is shown in Fig. 3. These results show that the total amount excreted in the urine following intravenous administration of 2.4, 4.8, and 9.6 mg of levodopa/kg was similar, indicating 60–65% of the administered dose. The patterns of urinary levodopa and its metabolites were essentially constant, independent of the levodopa dose among the doses administered.

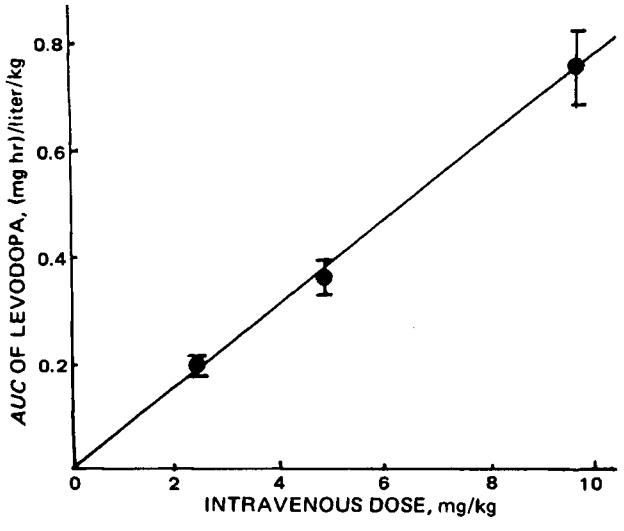

Figure 2—*Relationship between the intravenous dose and the observed* AUC *of levodopa corrected for body weight in dogs (average ± SE).*

[4] Total dopamine = conjugated dopamine + unconjugated dopamine.

Oral Administration of Levodopa in Dogs—The average plasma levels of levodopa and total dopamine following oral administration of 4.8, 9.6, and 19.2 mg of levodopa/kg to six dogs are shown in Fig. 4. As the levodopa dose administered increased, plasma levels of levodopa appeared to be disproportionally high. However, total dopamine, one of its metabolites, revealed an inverse situation; as the dose increased, the relative plasma levels of total dopamine were progressively lower. The average dose-normalized peak concentrations for levodopa were 0.11 ± 0.01, 0.14 ± 0.02, and 0.18 ± 0.01 kg/liter. While the differences do not appear to be statistically significant in most cases, they reflect a trend. The relationship between the levodopa dose administered and the ob-

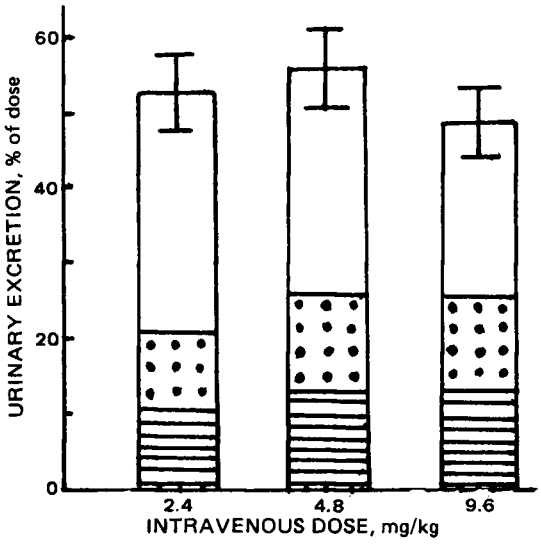

Figure 3—*Average urinary excretion of levodopa and its metabolites following intravenous administration of 2.4, 4.8, and 9.6 mg of levodopa/kg to six dogs. Key:* ▩, *total levodopa;* ■, *total dopamine;* ▨, *total 3,4-dihydroxyphenylacetic acid; and* □, *total homovanillic acid.*

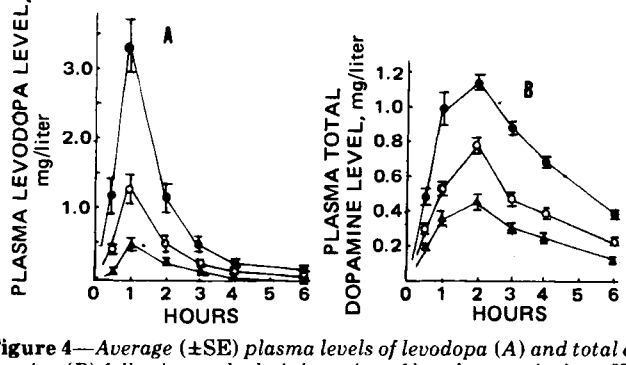

Figure 4—*Average* (±SE) *plasma levels of levodopa (A) and total dopamine (B) following oral administration of levodopa to six dogs. Key:* ▲, *4.8 mg/kg;* ○, *9.6 mg/kg; and* ●, *19.2 mg/kg.*

Table II—Average (±*SE*) Pharmacokinetic Parameters of Levodopa and *AUC* of Levodopa and Total Dopamine following Oral Administration of 4.8, 9.6, and 19.2 mg of Levodopa/kg to Six Dogs

Dose, mg/kg	Pharmacokinetic Parameters: k_{el}[a], hr^{-1}	Pharmacokinetic Parameters: $t_{1/2}$, hr	*AUC* of Levodopa Corrected for Weight, (mg hr)/liter/kg	*AUC* of Total Dopamine Corrected for Weight, (mg hr)/liter/kg	Ratio of *AUC* for Total Dopamine to Levodopa
4.8	0.97 ± 0.09	0.70 ± 0.07	0.080 ± 0.010	0.158 ± 0.013	1.79 ± 0.18
9.6	0.88 ± 0.11	0.74 ± 0.08	0.208 ± 0.024	0.299 ± 0.029	1.47 ± 0.14
19.2	0.96 ± 0.10	0.71 ± 0.07	0.478 ± 0.051	0.548 ± 0.051	1.09 ± 0.11

[a] Elimination rate constant of levodopa obtained by fitting the data to the one-compartment open model.

Table III—Average Urinary Excretion of Levodopa and Its Metabolites as Percentage of Administered Dose following Oral Administration of 4.8, 9.6, and 19.2 mg of Levodopa/kg to Six Dogs

Dose, mg/kg	Percent Dose Excreted in 0–48-hr Urine (Average ± *SE*): Total Levodopa[a]	Total Dopamine	Total 3,4-Dihydroxyphenylacetic Acid[b]	Total Homovanillic Acid[c]	Total[d]
4.8	0.45 ± 0.04 [e]	10.2 ± 1.0	10.1 ± 1.1	31.5 ± 3.1	52.5 ± 4.9
9.6	0.54 ± 0.05 [e]	12.8 ± 1.1	12.3 ± 1.2	29.5 ± 3.0	55.1 ± 5.1
19.2	0.64 ± 0.06 [e] [f]	12.5 ± 1.2	12.9 ± 1.4	24.5 ± 2.1	50.6 ± 4.5

[a] Total levodopa = unconjugated levodopa + conjugated levodopa. [b] Total 3,4-dihydroxyphenylacetic acid = unconjugated 3,4-dihydroxyphenylacetic acid + conjugated 3,4-dihydroxyphenylacetic acid. [c] Total homovanillic acid = unconjugated homovanillic acid + conjugated homovanillic acid. [d] Sum of total levodopa, total dopamine, total 3,4-dihydroxyphenylacetic acid, and total homovanillic acid. [e] Not significant ($p > 0.05$). [f] Significant ($p < 0.05$).

served *AUC* of levodopa is shown in Fig. 5. As the dose increased, the *AUC* increased disproportionately.

Elimination rate constants of levodopa after oral administration to dogs were calculated for each dog by fitting the data to the one-compartment open model. These values are summarized in Table II, along with the *AUC* of levodopa and total dopamine corrected for body weight and the ratio of the *AUC* of total dopamine to that of levodopa. The estimated elimination half-lives were essentially constant, independent of the levodopa dose, but the ratio of the *AUC* decreased with an incease in the dose. On the other hand, the average urinary excretion of levodopa and its metabolites over 48 hr after oral administration of 4.8, 9.6, and 19.2 mg of levodopa/kg to six dogs is shown in Table III. The total amount excreted in the urine following oral administration of the three different doses was similar, but the total levodopa excreted in the urine appeared to increase as the dose increased; only the differences between the 4.8- and 19.2-mg/kg doses were statistically significant ($p < 0.05$).

The absolute bioavailability and the total amount absorbed from the GI tract, including levodopa and its metabolites, following oral administration of 4.8, 9.6, and 19.2 mg of levodopa/kg to six dogs were calculated, based on the assumed linear kinetics, using the respective *AUC* or urinary excretion from intravenous doses of 4.8 and 9.6 mg/kg and also using the dose-corrected *AUC* or urinary excretion from an intravenous 19.2-mg/kg dose (Table IV). The absolute bioavailability of oral levodopa in dogs was dose dependent, while the total amount absorbed was essentially constant, independent of the dose administered.

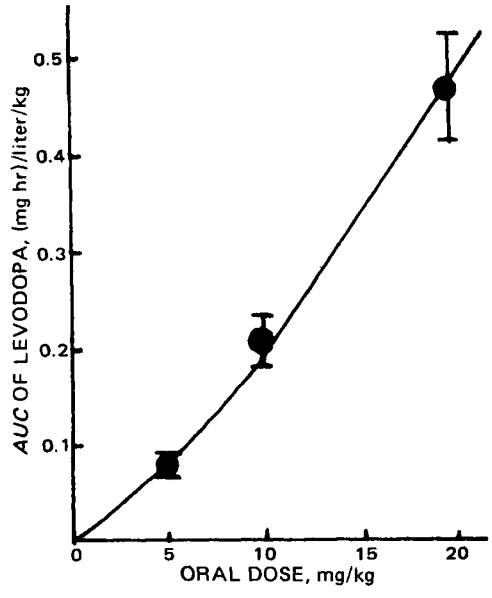

Figure 5—*Relationship between the oral dose and the observed* AUC *of levodopa corrected for body weight in dogs (average ± SE).*

Oral Dose Studies of Levodopa in Parkinsonian Patients—The average plasma levels of levodopa and total dopamine following oral administration of 3.8, 7.7, and 15.4 mg of levodopa/kg to three patients are shown in Fig. 6. The relationship between the dose administered and the plasma levels of levodopa and total dopamine was not directly proportional to the dose. As the dose increased, the relative plasma levels of levodopa were progressively higher but the relative plasma levels of total dopamine were progressively lower: *i.e.*, the dose-normalized plasma levels of levodopa increased with the dose while that of total dopamine decreased with the dose. The relationship between the levodopa dose and the *AUC* of levodopa is shown in Fig. 7. As found with the dogs, the *AUC* value of levodopa showed a nonlinear relationship as the dose increased.

The elimination rate constant of levodopa after oral administration to patients was calculated for each patient by fitting the data to a one-compartment open model. In addition, the ratio of the *AUC* of total dopamine to that of levodopa was calculated (Table V). The *AUC* values of levodopa and total dopamine corrected for body weight also are shown. The *AUC* of levodopa was constant and independent of the levodopa dose, but the *AUC* of total dopamine decreased with an increase in the dose.

The absolute bioavailability of doses of ~3.8, 7.7, and 15.4 mg of levodopa/kg after oral administration to three patients was evaluated on the assumptions that levodopa in the body obeys linear kinetics and that it is appropriate to compare the two different population groups, using the dose-corrected *AUC* from the intravenous dose reported previously (2). This result is listed in Table VI. The absolute bioavailability of oral levodopa in three different doses in patients was dose dependent in the same way as in dogs.

DISCUSSION

To evaluate the influence of the dose of levodopa on the pharmacoki-

Table IV—Absolute Bioavailability and Total Amount Absorbed, Including Metabolites, after Oral Administration to Six Dogs

Dose, mg/kg	Absolute Bioavailability, %	Total Amount Absorbed, %
4.8	22.1 ± 2.2[a]	81.7 ± 8.2[a]
9.6	27.7 ± 2.6[a]	93.1 ± 9.4[a]
19.2	31.9 ± 3.2[b]	78.1 ± 7.5[b]

[a] Based on the assumed linear kinetics using the respective *AUC* or urinary excretion from intravenous dosing (average ± *SE*). [b] Based on the assumed linear kinetics using the dose-corrected *AUC* or urinary excretion from intravenous dosing (average ± *SE*).

Table V—Average (±*SE*) Pharmacokinetic Parameters of Levodopa and *AUC* of Levodopa and Total Dopamine following Oral Administration of Doses of ~3.8, 7.7, and 15.4 mg of Levodopa/kg to Three Patients

Dose, mg/kg	Pharmacokinetic Parameters		*AUC* of Levodopa Corrected for Body Weight, (mg hr)/liter/kg	*AUC* of Total Dopamine Corrected for Body Weight, (mg hr)/liter/kg	Ratio of *AUC* for Total Dopamine to Levodopa
	k_{el}[a], hr^{-1}	$t_{1/2}$, hr			
3.8	0.88 ± 0.09	0.79 ± 0.08	0.008 ± 0.001	0.040 ± 0.003	4.81 ± 0.11
7.7	0.91 ± 0.06	0.77 ± 0.05	0.026 ± 0.003	0.061 ± 0.006	2.62 ± 0.28
15.4	0.90 ± 0.08	0.77 ± 0.07	0.069 ± 0.007	0.105 ± 0.040	1.62 ± 0.61

[a] Elimination rate constant of levodopa obtained by fitting the data to the one-compartment open model.

Table VI—Absolute Bioavailability of Doses of ~3.8, 7.7, and 15.4 mg of Levodopa/kg after Oral Administration to Three Patients

Dose, mg/kg	Absolute Bioavailability[a], % (Average ± *SE*)
3.8	15.2 ± 1.3
7.7	23.4 ± 1.9
15.4	31.2 ± 3.9

[a] Based on the assumed linear kinetics using the dose-corrected *AUC* from the intravenous dose.

netic behavior and bioavailability of the drug, plasma levodopa and total dopamine, one of its main metabolites, and urinary excretion following intravenous and oral administration to dogs and parkinsonian patients were determined. These results indicated that the pharmacokinetic behavior of levodopa and its total urinary excretion, including its metabolites, following intravenous administration to dogs was essentially constant within the dose range tested. Although the total urinary excretion was constant within the dose range after oral administration to dogs, the bioavailability of oral levodopa was dose dependent in both dogs and patients, and the urinary total dopamine also appeared to be dose dependent in dogs.

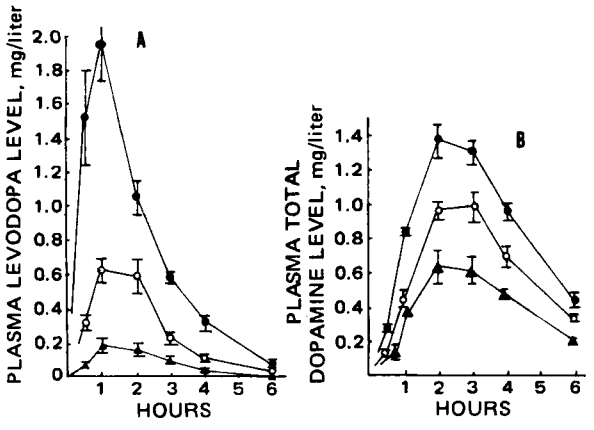

Figure 6—*Average* (±SE) *plasma levels of levodopa* (*A*) *and total dopamine* (*B*) *following oral administration of levodopa to three patients. Key:* ▲, *3.8 mg/kg;* O, *7.7 mg/kg; and* ●, *15.4 mg/kg.*

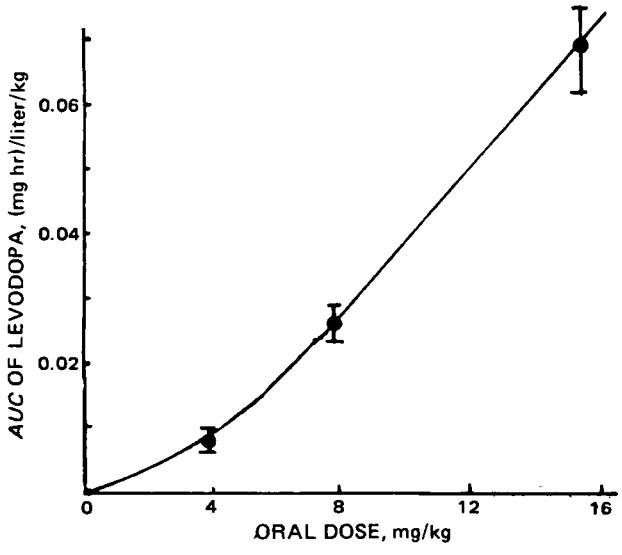

Figure 7—*Relationship between oral doses and observed* AUC *of levodopa in patients* (*average* ± SE).

The absolute bioavailability of oral levodopa must be calculated based on the assumption that levodopa in the body obeys linear kinetics. To test this assumption, an experiment is needed in which radiolabeled levodopa is given intravenously and unlabeled levodopa is given orally to the same subject at the same time. However, this assumption might be true since the clearance of levodopa in the plasma following intravenous administration proved to be constant, independent of the dose, and the elimination rate constant of levodopa in the plasma after oral administration was unchanged within the dose range tested, with nearly the same value found after intravenous administration. Furthermore, the change in total levodopa excreted in the urine after oral administration was proportional to the increase in the fraction of oral levodopa absorbed.

Bioavailability of the drug following oral administration is controlled by at least two factors: the rate and extent of uptake of levodopa in the gut and first-pass metabolism as a function of the dose. If active transport is the main process controlling the bioavailability of levodopa, then the total amount excreted in the urine can be expected to decrease as the dose increases. The observed results are contrary to this expectation. Therefore, if the active transport of levodopa in the GI tract occurs within the dose range tested, it does not play a significant role in affecting bioavailability. The elimination rate constant of levodopa in the plasma was observed to be constant, independent of the oral levodopa dose. This finding suggests the absence of saturation in the systemic elimination as the levodopa dose increased. In addition, the ratio of the *AUC* of total dopamine to that of levodopa after oral administration was small as the dose increased. This ratio reflects the extent of presystemic metabolism when compared to the ratio after intravenous administration (2).

These data support the contention that the dose-dependent bioavailability of levodopa is due to saturable first-pass metabolism during absorption. As the oral dose of levodopa is increased, the fraction of the dose metabolized to dopamine is reduced, and a larger fraction of the oral dose reaches the systemic circulation as intact, pharmacologically active drug. This phenomenon seems to be the most important factor in new dosage form design for improvement of the bioavailability of levodopa.

These results are interesting when they are related to the clinical use of levodopa. Hornykiewicz's assertion (10) that Parkinson's disease could be improved by offsetting the cerebral dopamine deficiency in parkinsonian patients with the administration of levodopa was not achieved at low doses of the drug. However, the high-dose therapy administered in 1967 by Cotzias *et al.* (11) led to the first successful treatment of Parkinson's disease and several subsequent studies (6, 12–14) supported their findings. The results in this report help explain these results.

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Simultaneous Determination of Cephalexin and Lysine in Their Salt Using High-Performance Liquid Chromatography of Derivatives

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Received November 2, 1979, from the *Analytical Department, Laboratorios Almirall, C/Cardoner, 68-74, Barcelona 24, Spain.* Accepted for publication February 29, 1980.

Abstract □ A sensitive and useful high-performance liquid chromatographic method using derivatization was developed for the simultaneous determination of intact cephalexin and lysine in their salt. This method is rapid and reliable, and its inherent specificity makes it an improvement over the common wet chemical methods for stability studies.

Keyphrases □ Cephalexin—simultaneous high-performance liquid chromatographic determination with lysine in their salt using derivatization □ Lysine—simultaneous high-performance liquid chromatographic determination with cephalexin in their salt using derivatization □ High-performance liquid chromatography—analysis, cephalexin and lysine in their salt, derivatization

The sodium salt of cephalexin is absorbed poorly after intramuscular injection (1, 2). Similar observations were made with a related compound, cephradine (3). Investigations with other soluble salts of cephalexin showed that some basic amino acids increased the absorption and bioavailability of cephalexin (4). The extensive use of the lysine salt of cephalexin necessitated the development of methods for the simultaneous determination of both components in this salt. Alkalimetric determination of carboxylic groups and nonaqueous titration of α-amino groups cannot be used because these two groups are present in both components. For the same reason, the Folin reaction with sodium β-naphthoquinone-4-sulfonate or hypobromite and α-naphthol (5) and the Saxena method (6) for microtitration of amino acid mixtures are unsatisfactory, and the selective titration of the ϵ-amino group of lysine in aqueous media with sulfuric acid is insufficiently accurate.

A recent report described the intramolecular aminolysis of some cephalosporins, caused by the nucleophilic attack of an α-amino group in the C-7 side chain in the β-lactam nucleus, which yields piperazine-2,5-dione derivatives (7). Estimation of the rate of disappearance of free amino groups was carried out by the 2,4,6-trinitrobenzenesulfonic acid (I) assay of Satake *et al.* (8) in a modified form. Applications of this technique to cephalexin lysinate was not possible due to interference by lysine.

A high-performance liquid chromatographic (HPLC) procedure for the analysis of lysine and cephalexin trinitrophenyl derivatives (II and III, respectively) is presented here. It is a rapid, sensitive, and specific method for the simultaneous determination of these two substances.

EXPERIMENTAL

Apparatus—Absorption spectra were obtained with a double-beam spectrophotometer[1] with a 1.0-cm cell. HPLC assays were performed with a liquid chromatograph[2] equipped with a variable-wavelength detector[3] and a 1-μl loop injection valve[4]. A stainless steel column (25 × 0.46 cm) loaded with alkylamine[5] (13 μm) was used.

Reagents—Cephalexin monohydrate[6], lysine hydrochloride[7], the lysine salt of cephalexin[8], and trinitrobenzenesulfonic acid[9] were used. The buffer solutions of pH values up to 7.6 were McIlvaine's buffers (9), the pH 7.6 buffer was prepared according to the method of Bundgaard (7), and the buffer solutions of higher pH were those of Clark and Lubs (10). All other reagents were analytical reagent grade.

General HPLC Procedure—An aqueous solution containing the lysine salt of cephalexin (2.0 ml) (5×10^{-4}–2×10^{-3} *M*) was mixed in a 100-ml volumetric flask with buffer solution (pH 10, 2.0 ml) and I (0.4% in water, 5.0 ml). The mixture was shaken and kept in the dark for 1 hr. Acetate buffer (pH 4.8, 20.0 ml) and aqueous 0.1% *o*-nitrophenol (2.0 ml as the internal standard) were added, and the solution was brought to volume with water.

A sample of this solution (1 μl) was injected into the liquid chromatograph and developed using an eluent system of 1% citric acid in a methanol–water mixture (5:40 v/v) with an isocratic flow rate of 0.75

[1] Perkin-Elmer 323.
[2] Perkin Elmer 601.
[3] Perkin-Elmer LC-55.
[4] Chrompack.
[5] Amino Sil-X-I.
[6] Lilly.
[7] Merck.
[8] Laboratorios Almirall.
[9] Aldrich.

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