Teratogenicity of Zinc Chloride, 1,10-Phenanthroline, and a Zinc–1,10-Phenanthroline Complex in Mice

CHI-HSIUNG CHANG *, DAVID E. MANN, Jr. ˣ, and RONALD F. GAUTIERI

Abstract □ Zinc chloride, in single doses of 12.5, 20.5, and 25 mg/kg ip on Day 8, 9, 10, or 11 of gestation in CF-1 albino mice, produced skeletal anomalies without accompanying soft tissue defects. Ripple ribs, the most unusual anomaly, first appeared when the zinc salt was given on Day 9 of gestation in a dose of 20.5 mg/kg, becoming more prevalent when 25 mg/kg of the drug was administered on Day 11. 1,10-Phenanthroline, in single doses of 30 mg/kg ip on Day 8, 9, 10, or 11 of gestation, elicited skeletal defects comparable to those caused by zinc chloride as well as soft tissue anomalies, but a significant incidence of the former occurred with this agent only following its injection on Day 8 of gestation. A zinc–1,10-phenanthroline complex in single doses of 50 mg/kg on Day 8, 9, 10, or 11 of gestation yielded significant incidences of skeletal and soft tissue anomalies only when the complex was administered on Day 8 or 9 of gestation. This dosage level was toxic to both the mother and fetus when given on Day 10 of gestation. However, when the complex was given on Day 8, 9, 10, or 11 in a dose of 25 mg/kg, neither toxic nor teratogenic effects were observed in the mother or fetus, respectively.

Keyphrases □ Zinc chloride—alone and complexed with 1,10-phenanthroline, teratogenic effects evaluated in mice □ 1,10-Phenanthroline—alone and complexed with zinc, teratogenic effects evaluated in mice □ Teratogenicity—zinc chloride and 1,10-phenanthroline, alone and complexed, mice □ Metals—zinc as chloride, alone and complexed with 1,10-phenanthroline, teratogenic effects evaluated in mice □ Chelating agents—1,10-phenanthroline, alone and complexed with zinc, teratogenic effects evaluated in mice

The importance of zinc for biological (botanical) growth was first recognized by Raulin (1), who discovered that it was essential for the development of *Aspergillus niger*. Lutz (2), after finding traces of the element in every organ of the cat, rat, and human, concluded that zinc was indispensable for zoological growth as well. Presently, the role of zinc in human nutrition has not been elucidated completely, although evidence has accumulated that it is necessary for nucleic acid synthesis and cell division (3–7).

Recent studies (8, 9) revealed that zinc is a limiting nutrient among members of village populations; in its absence, growth retardation and hypogonadism occur. Few reports pertain to the teratogenic effects arising from zinc supplementation and deprivation in mammals. Dietary studies in lower species have, however, shed some light on the results of zinc deprivation on fetal development. Hurley and Swenerton (10), after feeding a purified diet lacking zinc to pregnant rats, observed that all of the full-term fetuses had a wide variety of gross skeletal and soft tissue defects. Utilizing a different approach to determine the consequences of zinc deficiency in rodents, these investigators administered a chelating agent (edetic acid) to pregnant rats and succeeded in impairing reproduction and inducing congenital malformations (11). When this agent was fed to gravid rats on Days 6–21 of gestation, all full-term fetuses developed gross deformities, the recurrence of which was prevented in a later study identical to the first except for the supplementation of 1000 ppm of dietary zinc.

From these studies, it was assumed that zinc deficiency is a major etiologic factor in the occurrence of fetal anomalies in rodents. It was recently demonstrated (12) that zinc challenge also can cause congenital malformations in rodents; the injection of 2 mg of zinc sulfate/kg iv in pregnant golden hamsters resulted in teratogenic responses in the offspring.

Because of these results and the prevalence of malnutrition throughout the world along with the threat of widespread environmental pollution, this study attempted to verify the effects of deprivation and excessive challenge of zinc in another rodent species (mouse) by using a different chelating agent (1,10-phenanthroline) and a different zinc salt (the chloride). Affirmative results would stress the urgency to perform comparable studies in higher mammals (primates) whose teratogenic responsiveness is more relevant to that of humans.

EXPERIMENTAL

CF-1 albino mice[1] were employed. The females were placed in groups of 25–30 in aggregate cages and were not mated until they weighed at least 24 g. Males were placed in individual metal cages[2], 12.5 × 15 × 10 cm, with a wire mesh front and floor. Excreta pans were elevated to the cage floor to permit coprophagy[3]. The animals were maintained on laboratory food[4] and tap water *ad libitum*.

To produce timed pregnancies, two female mice were placed in the cage of a fertile male at 4:00 pm. The following morning, at 8:00 am, the females were removed and inspected for the presence of a vaginal plug, which represents the last part of an ejaculate. Animals exhibiting this sign were considered to be gravid, and the morning of the appearance of the plug was designated as the start of gestation or Day 0. The gravid animals were then weighed and placed in individual cages, similar to those of the males, where they remained undisturbed until the morning of Day 7 when they were reweighed. Pregnancy was confirmed by a weight gain of 2 g or more.

Following the respective treatments, the mice were allowed to continue their gestation periods uninterrupted until Day 18, which was 1 day prior to the time of expected delivery. On this day, each pregnant mouse was sacrificed by cervical dislocation and its weight was recorded. The abdominal cavity was opened to expose the uterine horns. The number of fetuses and resorption sites (metrial glands) was determined and recorded. The exposed fetuses were removed by blunt dissection, blotted dry, and weighed to the nearest 0.01 g on a torsion balance. Each fetus was then examined for external defects and sexed.

Viability was ascertained by reflex movement of the limbs in response to mechanical stimulation with a blunt probe after removal of the fetus from the uterus. Every second fetus was processed for skeletal examination according to the method of Staples and Schnell (13). The remaining fetuses were fixed and decalcified in Bouin's fixative. After 2 weeks, the fixative was replaced by 70% ethyl alcohol in which the spec-

[1] Carworth Farms, New City, N.Y.
[2] Norwich Wire Works, Norwich, N.Y.
[3] To prevent skin lesions.
[4] Purina.

Table I—Skeletal Anomalies Induced by Zinc Chloride, I, and II on Days 8–11 of Gestation in CF-1 Albino Mice

Drug	Day of Gestation	Total Number of Fetuses	Number of Fetuses with Respective Defects	Total Number of Fetuses with Defects[a] / Total Number of Fetuses
Water	8	47	4, delayed ossification and fused sternebrae 2, supernumerary ribs 1, delayed ossification of xiphoid process	5/47 (10.6%)
Zinc chloride, 20.5 mg/kg	8	34	26, delayed ossification and fused sternebrae; crankshaft sternebrae 1, supernumerary ribs 13, delayed ossification of xiphoid 5, delayed ossification of hindpaws 2, delayed ossification of forepaws	26/34[b] (76.4%)
I, 30 mg/kg	8	53	14, delayed ossification and fused sternebrae; crankshaft sternebrae 2, supernumerary ribs 1, delayed ossification of xiphoid 1, delayed ossification of phalanges of hindpaws 1, delayed ossification of forepaws	16/53[b] (30.1%)
II, 50 mg/kg	8	20	15, delayed ossification and fused sternebrae; crankshaft sternebrae 3, supernumerary ribs 6, delayed ossification of xiphoid 2, delayed ossification of hindpaws 2, exencephaly	17/20[b] (85.0%)
Water	9	53	9, delayed ossification and fused sternebrae; crankshaft sternebrae 2, delayed ossification of xiphoid 2, delayed ossification of phalanges of hindpaws 2, delayed ossification of phalanges of forepaws 1, exencephaly	9/53 (16.9%)
Zinc chloride, 20.5 mg/kg	9	48	16, delayed ossification and fused sternebrae; supernumerary and crankshaft sternebrae 10, supernumerary and ripple ribs 6, ripple ribs 12, delayed ossification of xiphoid 6, delayed ossification of phalanges of hindpaws 6, delayed ossification of phalanges of forepaws	19/48[b] (39.5%)
II, 50 mg/kg	9	19	17, fused vertebrae 7, delayed ossification and crankshaft sternebrae 10, fused or missing ribs 6, delayed ossification of xiphoid 1, delayed ossification of phalanges of hindpaws 2, delayed ossification of phalanges of forepaws	18/19[b] (94.7%)
Water	10	38	2, fused and crankshaft sternebrae 4, supernumerary ribs 1, delayed ossification of xiphoid 1, delayed ossification of phalanges of hindpaws 1, delayed ossification of phalanges of forepaws	6/38 (15.7%)
Zinc chloride, 20.5 mg/kg	10	30	8, delayed ossification of sternebrae 5, supernumerary and ripple ribs 4, ripple ribs 8, delayed ossification of xiphoid 7, delayed ossification of phalanges of hindpaws 10, delayed ossification of phalanges of forepaws	11/30[b] (36.6%)
Water	11	87	6, crankshaft and fused sternebrae; supernumerary ribs	7/87 (8.0%)
Zinc chloride, 20.5 mg/kg	11	79	23, crankshaft sternebrae 26, supernumerary and ripple ribs and missing ribs 21, ripple ribs 2, delayed ossification of phalanges of hindpaws 2, delayed ossification of phalanges of forepaws 1, exencephaly	38/79[b] (48.1%)
Zinc chloride, 25 mg/kg	11	39	12, delayed ossification of sternebrae 26, supernumerary and ripple ribs 24, ripple ribs 8, delayed ossification of xiphoid 18, delayed ossification of phalanges of hindpaws 5, delayed ossification of phalanges of forepaws	30/39[b] (76.9%)
II, 50 mg/kg	11	15	3, supernumerary and ripple ribs 2, ripple ribs 3, delayed ossification of phalanges of hindpaws	5/15[b] (33.3%)

[a] Total number of defects refers to total number of fetuses with skeletal anomalies. [b] Statistically different at level of $p < 0.05$ (compared to respective water-treated group).

imens were stored until freehand sections were made with a double-edged, stainless steel razor blade[5] according to the technique of Wilson (14).

Statistical Methods and Analysis—The statistical significance of variations among the experimental groups was estimated by the Student t test for continuous variates and the unadjusted χ-square or Yates' corrected χ-square for binomial proportions (15). Probabilities, p, less than 0.05 were considered to represent significant differences.

[5] Durham Duplex, Durham Enders Co., Mystic, Conn.

Treatment Regimen—Animals were divided into seven groups and received the following treatment regimens: zinc chloride, 12.5, 20.5, and 25 mg/kg (three groups); 1,10-phenanthroline (I), 30 mg/kg (one group); a zinc–1,10-phenanthroline complex (II), 25 and 50 mg/kg (two groups), and distilled water (one group). The number of litters ranged from six to 10 for each group. All injections were given intraperitoneally, utilizing aqueous preparations of each drug whose volume never exceeded 0.45 ml. The complex was prepared by mixing zinc chloride (2.045 mg/0.5 ml of distilled water) with 1,10-phenanthroline (3 mg/ml of distilled water).

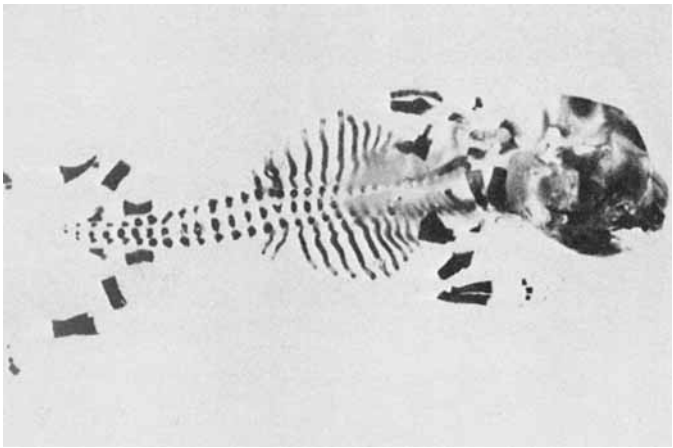

Figure 1—*Alizarin skeletal preparation of near-term mouse fetus from a female treated on Day 9 of gestation with 20.5 mg of zinc chloride/kg, showing ripple ribs.*

RESULTS

Zinc Chloride-Induced Teratogenesis—Zinc chloride, in a dose of 20.5 mg/kg on Day 8 of gestation, produced a 76.4% incidence of skeletal anomalies (Table I). Two out of seven gravid mice resorbed all of their fetuses. This dosage, when administered on Day 9 of gestation, yielded a 39.5% incidence of skeletal anomalies including the ripple rib, a thickening of the midportion of the rib with a peculiar undulation (Fig. 1). The ripple rib anomaly, which first appeared on Day 9 of gestation with a 12.5% occurrence in response to zinc chloride challenge, was accompanied by other skeletal defects similar to those caused by the zinc salt on Day 8.

When zinc chloride was given on Day 10 or 11 of gestation in a dose of 20.5 mg/kg, incidences of skeletal defects of 36.6 and 48.1%, respectively, were produced that were comparable to those appearing in response to the challenge by the zinc salt on Day 9 with respect to morphological alteration. This dose was most toxic when given on Day 10 of gestation; four out of 10 gravid mice died several days after having received single injections. The incidence of skeletal anomalies caused by zinc chloride on Days 8–11 of gestation was significantly higher than that of the water-treated group, but the occurrence of soft tissue anomalies in response to this dose of the zinc salt during the same period was not significant as compared to that of the water-treated group.

Zinc chloride, 25 mg/kg on Day 11 of gestation, produced an incidence of 76.9% of skeletal deformities, including a 61.5% occurrence of ripple ribs (Table I). The difference between the percent incidence of skeletal anomalies caused by this dose of the zinc salt and that of the water-treated group was significant. This dose of zinc chloride elicited the greatest incidence of maternal deaths; five out of 15 gravid mice died when the injection was made on Day 11 of gestation.

Zinc chloride, 12.5 mg/kg on Day 11 of gestation, induced nonsignificant incidences of both skeletal and soft tissue defects as compared to those of the water-treated group. No deaths were observed in the gravid animals, and no ripple ribs were noted in the fetuses.

Compound I-Induced Teratogenesis—Compound I, 30 mg/kg, produced a 30.1% incidence of skeletal defects (Table I) when administered on Day 8 of gestation and a 13.0% incidence of soft tissue anomalies (Table II) when given on Day 9. Both percent incidences were significantly different from those of the water-treated group.

No significant differences in the incidence of skeletal and soft tissue anomalies were noted between the I and water-treated groups when administrations were given on Days 10 and 11. Four fetuses out of 10 litters and 13 fetuses out of seven litters were resorbed when this dose of I was given on Days 10 and 11, respectively. No maternal deaths occurred following the administration of I on Days 8–11 of gestation.

Complex II-Induced Teratogenesis—Complex II, 50 mg/kg on Day 8, produced an incidence of skeletal anomalies of 85.0%. This dose of the complex, when given on Day 9 of gestation, produced a 94.7% incidence of skeletal defects, including fused vertebrae. The latter malformation was initially observed when the complex was injected on Day 9 at this dosage level. Interestingly, neither the zinc salt nor I elicited the anomaly, regardless of the day of injection.

A number of soft tissue defects, including extra thumbs and missing digits and tail, were caused by the complex. Both the incidences of skeletal anomalies (94.7%) and soft tissue defects (23.5%) were significantly higher than those of the water-treated group that received injections on Day 9 (Tables I and II). A high incidence of maternal deaths was observed a few days after II was administered. Seven out of 14 gravid animals died, and two out of 14 gravid mice resorbed all of their fetuses. Twenty-one fetal resorptions in seven litters occurred after the injection of the complex on Day 9.

Table II—Soft Tissue Anomalies Induced by I and II on Day 8 or 9 of Gestation in CF-1 Albino Mice[a]

Drug	Day of Gestation	Total Number of Fetuses	Number of Fetuses with Respective Defects	Total Number of Fetuses with Defects[b] / Total Number of Fetuses
Water	8	43	3, sixth digit[c] 1, cleft palate	4/43 (9.3%)
II, 50 mg/kg	8	18	1, sixth digit 2, exencephaly 1, cleft palate 3, cryptorchidism	7/18 (38.9%)[d]
Water	9	46	1, cryptorchidism	1/46 (2.2%)
I, 30 mg/kg	9	46	3, sixth digit 1, cleft palate 1, talipes 1, cryptorchidism	6/46 (13.0%)[d]
II, 50 mg/kg	9	17	4, two thumbs; four digits 1, tail absent	4/17 (23.5%)[d]

[a] No significant defects of soft tissues were noted on Days 10 and 11 of gestation when zinc chloride, I, or half as much II was administered individually to mice. Neither the injection of zinc chloride nor half as much II on Day 8 or 9 of gestation produced a significant incidence of soft tissue anomalies. [b] Total number of defects refers to total number of fetuses with soft tissue anomalies. [c] Actually is a protuberance adjacent to fifth digit of forepaw. [d] Statistically different at level of $p < 0.05$ (compared to water-treated group).

The administration of II on Day 10 of gestation, in a dose of 50 mg/kg, proved to be highly toxic to both the mother and fetus. Five of 14 gravid animals succumbed, and eight of 14 mice resorbed all of their fetuses. Nine fetuses in nine litters were resorbed. Obviously, these conditions preempted the study of skeletal and soft tissue defects.

Complex II, 50 mg/kg on Day 11 of gestation, produced a 33.3% incidence of skeletal anomalies (Table I), including ripple ribs which had not been noted when the complex was injected on earlier gestation days at this dosage level. The incidence of skeletal defects (33.3%) was significantly higher than that observed in the water-treated group. Only one in eight gravid mice resorbed all of her fetuses, but the maternal death rate was high (50%). Fourteen fetuses in four litters were resorbed.

The single administration of II, in a dose of 25 mg/kg on Days 9–11, yielded nonsignificant incidences of skeletal and soft tissue defects as compared to those of the water-treated groups. Maternal deaths and total fetal resorptions were not elicited by this dose of the complex.

DISCUSSION

Zinc chloride, when administered intraperitoneally to CF-1 albino mice in doses of 20.5 and 25 mg/kg, produced significant incidences of skeletal defects as compared to those observed in the water-treated group on Day 11. However, the zinc salt failed to produce a significant incidence of soft tissue anomalies with this treatment regimen. As the dosage of the zinc salt was reduced, maternal and fetal toxicity, relative fetal weights, and the incidence of skeletal anomalies were correspondingly decreased. The principal site for the teratogenicity of the zinc salt apparently is the skeletal system and, with increased doses, the majority of the defects produced involve the rib cage (*i.e.*, ripple ribs).

Zinc chloride, 20.5 mg/kg, exerted greater toxic effects on the mother and fetuses when administered on Day 10 of gestation than on any other day. Ripple ribs (Fig. 1) caused by the zinc salt first appeared when the drug was given on Day 9 of gestation and became more frequent and pronounced after the injection on Day 11.

Compound I, in a dose of 30 mg/kg, produced a significant incidence of skeletal defects on Day 8 and soft tissue anomalies on Day 9. Maternal deaths were not observed when the chelating agent was injected on Days 8–11. Only one in 10 gravid mice resorbed all of her fetuses when I was given on Day 10. At this dose, the chelator exerted relatively little toxicity since the teratogenic effects occurred only when I was given on Day 8 or 9. If the majority of the defects arising from I injections were due to

chelation with endogenous zinc in a bound or free state, it could then be assumed that the metal requirements of fetuses on Days 8 and 9 of gestation are considerably higher or more important than the demands for the metal after Day 9.

Complex II, in a dose of 25 mg/kg, failed to elicit significant skeletal and soft tissue anomalies when injected on Days 8–11, suggesting that any lower dose would be unlikely to cause teratogenesis and toxicity in fetuses and maternal animals, respectively. A dose of 50 mg/kg of the complex did produce significant incidences of skeletal and soft tissue defects when given on Day 8 or 9. When the complex was administered in this dose on Day 10, increased toxicity to both gravid mice and fetuses overshadowed its teratogenic actions. Yet, when the complex was given on Day 11, skeletal instead of soft tissue anomalies appeared in significant incidences. Furthermore, although the zinc is bound with 1,10-phenanthroline when injected as a complex, sufficient amounts of the metal apparently are available to elicit teratogenic effects in a manner comparable to that achieved by free zinc since ripple ribs occurred only when large doses of the complex were given and in an incidence considerably lower than that induced by the intermediate dose (20.5 mg/kg) of the zinc salt on Days 11 and 9.

CONCLUSION

On the basis of these results utilizing lower species, it is recommended that similar studies be carried out in higher mammals to determine more precisely the relevancy of deprivation and excessive challenge of zinc as etiologic factors in the development of human teratogenesis.

REFERENCES

(1) J. Raulin, *Ann. Sci. Nat. Bot. Biol. Veg.*, 11, 93 (1869).

(2) R. E. Lutz, *J. Ind. Hyg.*, 8, 177 (1926).

(3) B. J. Poiesz, N. Battula, and L. A. Loeb, *Biochem. Biophys. Res. Commun.*, **56**, 959 (1974).

(4) M. C. Scrutton, C. W. Wu, and D. A. Goldthwait, *Proc. Natl. Acad. Sci. USA*, **68**, 2497 (1971).

(5) J. P. Slater, A. S. Mildvan, and L. A. Loeb, *Biochem. Biophys. Res. Commun.*, **44**, 37 (1971).

(6) C. F. Springate, A. S. Mildvan, R. Abramson, J. L. Engle, and L. A. Loeb, *J. Biol. Chem.*, **248**, 5987 (1973).

(7) B. L. Vallee, in "Advances in Protein Chemistry," M. L. Anson, V. Bailey, and J. T. Edsall, Eds., Academic, New York, N.Y., 1955, p. 317.

(8) A. S. Prasad, A. Miale, Jr., Z. Farid, H. H. Sandstead, and A. R. Schulert, *J. Lab. Clin. Med.*, **61**, 537 (1963).

(9) H. H. Sandstead, A. S. Prasad, A. R. Schulert, Z. Farid, A. Miale, Jr., S. Bassily, and W. J. Darby, *Am. J. Clin. Nutr.*, **20**, 422 (1967).

(10) L. S. Hurley and H. Swenerton, *Proc. Soc. Exp. Biol. Med.*, **123**, 692 (1966).

(11) H. Swenerton and L. S. Hurley, *Science*, **173**, 62 (1971).

(12) V. H. Ferm and S. J. Carpenter, *Nature*, **216**, 1123 (1967).

(13) R. E. Staples and V. L. Schnell, *Stain Technol.*, **39**, 61 (1964).

(14) J. G. Wilson, in "Teratology Principles and Techniques," J. G. Wilson and J. Warkany, Eds., University of Chicago Press, Chicago, Ill., 1965, p. 267.

(15) G. G. Simpson, A. Roe, and R. C. Lewontin, "Quantitative Zoology," Harcourt, Brace, New York, N.Y., 1960, pp. 186–195.

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* Present address: Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, NY 14203.

[x] To whom inquiries should be directed.

Determination of Time Course of Tablet Disintegration I: Numerical Method

K. G. NELSON [x] and L. Y. WANG

Abstract □ A method is described for determining the time course of tablet disintegration. It involves a numerical analysis of the experimental dissolution profile of a tablet and the dissolution characteristics of the primary drug particles in the tablet. The disintegration profile is determined for an acetaminophen tablet to demonstrate the application of the method. Tablet dissolution is simulated with the disintegration–dissolution model, and the interrelationship between the two fundamental processes is studied theoretically by varying the parameters describing the two processes.

Keyphrases □ Disintegration—tablets, time course determined using numerical analysis of dissolution profile □ Dissolution profile—numerical analysis used to determine time course of tablet disintegration □ Tablets—time course of disintegration determined using numerical analysis of dissolution profile

The release of an active ingredient from a tablet involves two distinct processes: disintegration of the tablet and dissolution of the active ingredient. Although both processes commence when the tablet encounters an aqueous environment, the bulk of the active ingredient cannot dissolve until disintegration has occurred. The two processes are thus sequential and occur simultaneously until the tablet has disintegrated completely.

Studies on the temporal aspect of disintegration have largely involved only one time point, the disintegration time. The disintegration time is a subjective measure involving the time it takes the tablet to disintegrate and pass through a screen of arbitrary size in a standard apparatus (1). Attempts at determining the time course of disintegration have been reported (2, 3), but generally research in this area has involved the study of parameters that affect disintegration such as disintegrant type, compression force, and binders, and the results have been monitored by the official disintegration test (4).

Dissolution processes for powders and nondisintegrating tablets have been studied rather extensively. Several reports discuss dissolution theory and provide mathematical models (5–8) which serve as a means to evaluate dissolution rate data and dissolution test systems. A dissolution theory for disintegrating tablets, however, has not been developed, although equations with adjustable parameters have described the process (9, 10).

This paper describes a method for processing dissolution rate data to determine a quantitative description of the disintegration process as a function of time. The method was applied to experimental dissolution rate data from a tablet having a rather idealized formulation to demon-