sumed by approximation to be constant (*cf.*, Eq. 5). The consequent proportionality between dQ/dt and dl/dt should enable derivation of Eq. 4 in complete analogy with Eq. 1. That the condition for proportionality is generally satisfied also for values of A as little as $3C_s$ can be verified by expressing this condition through the ratio of the first to the second term in the expression of the release rate (Eq. A3):

$$\left|\frac{2A - C_s - C_v}{l} dl/dC_v\right| \gg 1 \qquad \text{(Eq. A4)}$$

and further substituting into this relationship the following expressions of C_v, dC_v, l, and dl:

$$C_v = R\, dQ/dt \qquad \text{(Eq. A5)}$$

$$dC_v = (R\, d^2Q/dt^2)\, dt \qquad \text{(Eq. A6)}$$

$$l = Q/(n - 1/2)C_s \qquad \text{(Eq. A7)}$$

$$dl = [1/(n - 1/2)C_s]\, dQ \qquad \text{(Eq. A8)}$$

where $n = A/C_s$. Equation 1, where $(n - 1/2)C_s$ is used in place of A, can be employed to obtain approximate expressions of Q, dQ, and d^2Q/dt^2.

Following calculation, the condition for proportionality between the release rate and the rate of increase of the thickness of the depletion zone can be written as follows:

$$\frac{2x^2(n - 1/2) - x}{x - 1} \gg 1 \qquad \text{(Eq. A9)}$$

where $x = \sqrt{1 + [2/(n - 1/2)D_vR^2]t}$.

It can be readily verified that the minimum value of the ratio is 18 for $n = 3$, while it increases with increasing n.

REFERENCES

(1) T. Higuchi, *J. Soc. Cosmet. Chem.*, **11**, 85 (1960).

(2) W. I. Higuchi, *J. Pharm. Sci.*, **51**, 802 (1962).

(3) *Ibid.*, **56**, 315 (1967).

(4) *Ibid.*, **50**, 874 (1961).

(5) *Ibid.*, **52**, 1145 (1963).

(6) T. J. Roseman and W. I. Higuchi, *J. Pharm. Sci.*, **59**, 353 (1970).

(7) T. J. Roseman, *ibid.*, **61**, 46 (1972).

(8) A. L. Weiss and B. J. Sciarrone, *ibid.*, **58**, 980 (1969).

(9) C. W. Whitworth and R. E. Stephenson, *ibid.*, **60**, 48 (1971).

(10) J. Haleblian, R. Runkel, N. Mueller, J. Christopherson, and K. Ng, *ibid.*, **60**, 541 (1971).

(11) G. L. Flynn, S. H. Yalkowsky, and T. J. Roseman, *ibid.*, **63**, 487 (1974).

(12) "Proceedings of the Post-Graduate School on Diffusion in Pharmaceutical Formulation and Packaging," The Pharmaceutical Society of Great Britain, London, England, Apr. 1975, pp. 186–193.

(13) A. G. Perotti, *Farmaco, Ed. Prat.*, **25**, 651 (1970).

(14) F. Bottari, G. Di Colo, E. Nannipieri, M. F. Saettone, and M. F. Serafini, *J. Pharm. Sci.*, **63**, 1779 (1974).

(15) *Ibid.*, **64**, 946 (1975).

(16) F. Bottari, G. Di Colo, E. Nannipieri, M. F. Saettone, and M. F. Serafini, *Boll. Chim. Farm.*, **115**, 113 (1976).

(17) P. Singh, S. J. Desai, D. R. Flanagen, A. P. Simonelli, and W. I. Higuchi, *J. Pharm. Sci.*, **57**, 959 (1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 5, 1976, from the *Pharmaceutical Technology Laboratory, Institute of Pharmaceutical Chemistry, University of Pisa, 56100, Pisa, Italy.*

Accepted for publication August 10, 1976.

Supported in part by a grant from Consiglio Nazionale delle Ricerche.

Reference 14 is considered Part I of this series.

The authors thank Prof. Olga Dun De Finamore for assistance.

[x] To whom inquiries should be directed.

Metabolism of *N*-Ethyl-3-piperidyl Benzilate in Rats

CHING-HSIU CHEN [*], MAHMOUD M. ABDEL-MONEM [*x], and THOMAS P. KRICK [‡]

Abstract □ The metabolic fate of *N*-ethyl-3-piperidyl benzilate (I) and its potential metabolites 3-piperidyl benzilate (II), *N*-ethyl-3-hydroxypiperidine (III), and 3-hydroxypiperidine (IV) was studied. Incubation of I with rat liver homogenates resulted in the formation of II and III. Only a trace of unchanged drug appeared in urine after intraperitoneal injection of I. Approximately 9% of the injected dose of I was excreted in urine as III and 2% in the form of metabolites that produced III after acid hydrolysis. After intraperitoneal injection of II in rats, 18% of the dose was excreted in urine as IV. Approximately 26% of the injected dose of III was present in urine as the unchanged drug, and 63% of the dose was excreted in the urine in the form of conjugates that produced III on acid hydrolysis. Urine of rats injected with IV contained approximately 50% of the injected dose as the unchanged drug and 50% of the dose in the form of a conjugate that produced IV on acid hydrolysis. The identity of the metabolites in extracts from urine was established by GLC–mass spectrometry. It is concluded that hydrolysis was one metabolic pathway for I and II. The major routes of elimination of these compounds are not yet known and may include excretion in feces or metabolic transformations resulting in the degradation of the piperidine ring.

Keyphrases □ *N*-Ethyl-3-piperidyl benzilate—metabolism in rats □ Metabolism—*N*-ethyl-3-piperidyl benzilate in rats □ Benzilates—*N*-ethyl-3-piperidyl ester, metabolism in rats

During studies to develop potent and selectively acting atropine substituents for the treatment of GI disturbances, numerous compounds were synthesized that were superior to atropine with respect to anticholinergic potency, antisecretory effects, and reduced incidence of side effects (1). Most of these compounds are glycolate esters of heterocyclic imino alcohols. In experimental animals, these drugs produced potent central nervous system (CNS) stimulation, as indicated by hyperactivity and other behavioral disturbance tests (2). In humans, they were potent and long-lasting hallucinogenic and psychotomimetic agents (3).

Some human subjects manifested a marked change in their basic mood and drive 1 or 2 days following a single

dose of these drugs (4). Clinical trials of one compound[1] in psychiatric patients revealed that it was a safe and effective antidepressant agent (5). The delayed onset of the mood enhancement effects of the glycolate esters in humans might be attributed to the formation of active metabolites. Therefore, the metabolic fate of these compounds in rats was studied as a prelude to metabolic studies in humans.

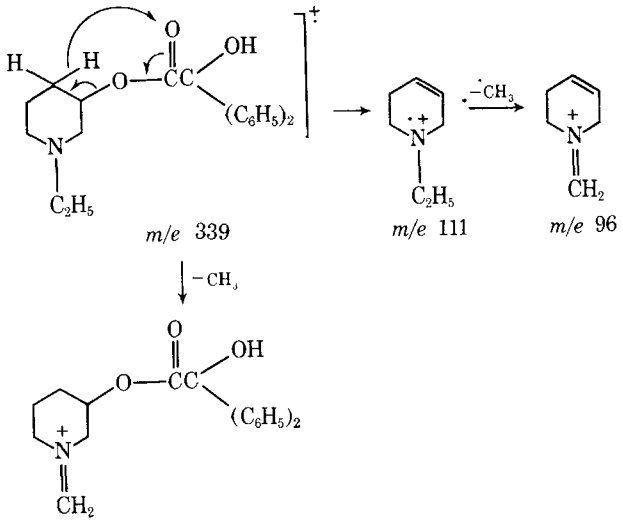
The metabolic fate of glycolate esters has received little attention, and the few reported studies provide somewhat contradictory results. The metabolism of ^{3}H-*N*-ethyl-3-piperidyl benzilate (I) was studied in rats; 75% of the injected dose was present in urine as the unchanged drug at 2 hr after intraperitoneal injection, and 96% was excreted after 12 hr (6). However, the unchanged drug was not detected in urine of rats during 48 hr after intraperitoneal injection of I and the only metabolites detected were benzilic acid, *N*-ethyl-3-hydroxypiperidine (III), and a small amount of conjugate(s) that produced III after acid hydrolysis (7). Another study (8) found that I produced acetaldehyde when incubated *in vitro* with microsomes from rat liver. The present paper reports the identification of the metabolites produced from the incubation of I with rat liver homogenate *in vitro* and the urinary metabolites of I after intraperitoneal injection in rats.

RESULTS AND DISCUSSION

Synthesis and Identification of Potential Metabolites—The hydrochloride of I was synthesized from III and methyl benzilate as described by Biel *et al.* (1). 3-Piperidyl benzilate (II) hydrochloride was obtained by catalytic hydrogenolysis of *N*-benzyl-3-piperidyl benzilate. The latter was obtained by reacting *N*-benzyl-3-chloropiperidine with benzilic acid. The hydrochlorides of III and 3-hydroxypiperidine (IV) were obtained from commercially available III and IV.

***In Vitro* Studies**—Compound I hydrochloride was incubated with rat liver homogenate. The metabolites were extracted from the incubation mixture and examined by two-dimensional TLC on silica gel plates. Spots with chromatographic mobilities similar to authentic samples of I–III were observed on the TLC plates. No spot corresponding in position to IV was observed.

To confirm the identity of the metabolites produced, areas of the TLC plates corresponding to the positions of I–III were scraped and the adsorbent was extracted with methanol. The methanol extracts were evaporated to dryness, and the residues were submitted for mass spectral analysis. The mass spectrum of an authentic sample of I and that of the substance recovered from the incubation mixture were virtually identical, confirming the identity of the latter. The mass spectrum for I showed the molecular ion at *m/e* 339. The base peak appeared at *m/e* 111 and was tentatively assigned to the $C_7H_{13}N^+$ fragment. A plausible fragmentation pathway accounting for the major fragments in the mass spectrum of I is shown in Scheme I.



Scheme I

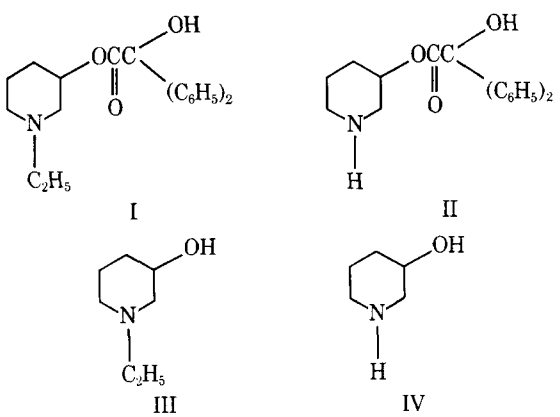
The mass spectrum of authentic II was not identical to that of the metabolite believed to be II. Evidently, the fragmentation pattern of II by electron impact was dependent on the concentration of the compound and/or the temperature in the ionization chamber, since recording consecutive spectra after the introduction of the sample in the ionization chamber of the mass spectrometer resulted in vastly different spectra. To confirm the identity of the metabolite, the IR spectrum of the residue eluted from the TLC plate was recorded and was identical to that of an authentic sample of II.

The crude extract from the incubation mixture was examined by GLC–mass spectrometry to detect the presence of III and IV. The chromatographic separation was performed on a 12% Carbowax 20M column, and the mass spectrum for the peaks eluted with the same retention time as III and IV were recorded. No peak with the same retention time and mass spectrum as IV was detected in the crude extract of the incubation mixture. A peak with the same retention time as III was present in this extract, and the mass spectrum of this peak was virtually identical to that obtained for an authentic sample of III. The mass spectrum of III showed the molecular ion at *m/e* 129 and the base peak at *m/e* 114 and was similar to the spectrum previously reported for this compound (9).

The possibility that III was produced by the nonenzymatic hydrolysis of I also was explored. Incubation of I under the same conditions as for the enzymatic studies, except for the absence of liver homogenate, resulted in the formation of only a trace of III, if at all. This result indicated that the hydrolysis of I to III in the previous studies was indeed enzyme catalyzed, undoubtedly by esterases present in liver homogenates. It was of interest to determine the site of these esterases in the different fractions of liver homogenates and whether these esterases were present in rat blood.

Compound I was incubated with the microsomal and the supernatant fractions of the rat liver homogenate and with rat blood. The incubation conditions and the workup of the incubation mixture were the same as described for the experiments using liver homogenates. Incubation of I with the microsomal fraction of the rat liver homogenates resulted in a metabolite profile qualitatively and quantitatively (as determined by comparison of the relative intensities of the spots on the TLC plate) similar to that obtained with the rat liver homogenates. On the other hand, incubation of I with the 105,000×*g* supernate of rat liver homogenates resulted only in the formation of traces of III. The formation of III by incubation with the 105,000×*g* supernate may be attributed to the presence of residual microsomes or the presence of esterase enzymes in this fraction. Incubation of I with rat blood resulted in the formation of traces of III. These results indicated that the enzymes responsible for the metabolism of I *in vitro* were mainly localized in the microsomal fraction of the liver homogenates. The blood showed only a very low esterase activity for this compound.

The absence of IV in the products of the metabolism of I was not expected, since the incubation conditions resulted in the accumulation of II and III in the incubation mixture. The latter two metabolites would be expected to be substrates for the liver enzymes and would be further metabolized to IV. Therefore, II and III were investigated as substrates



[1] Ditran.

for the rat liver homogenate enzymes. Compounds II and III were incubated with the rat liver homogenates using the same incubation conditions and workup procedures described for I. These studies indicated that II was not hydrolyzed to IV by the microsomal esterases. Likewise, III was not *N*-deethylated to IV.

Urinary Metabolites of I in Rats—The urinary excretion of I and its potential metabolites after intraperitoneal injection in rats also was studied. Several methods for the isolation of the metabolites from urine were investigated, including extraction of urine with solvents, adsorption of the metabolites on activated charcoal (10), use of cation-exchange resins to retain the basic metabolites, and partition column chromatography as described by Egli (7). The use of activated charcoal for the adsorption of the metabolites from urine was most satisfactory. The recovery of metabolites from urine was high, and there was minimum interference from other urinary constituents.

Extracts from urine of rats injected with I hydrochloride were examined by GLC–mass spectrometry using a 3% OV-1 column and monitoring *m/e* 339 and 129. A peak of *m/e* 339, at a column temperature of 220°, with the same retention time as I was observed in the urine extracts, indicating the urinary excretion of unchanged drug. The identity of this substance was further established by recording the spectrum of this peak between *m/e* 400 and 10. This spectrum was virtually identical to that obtained for an authentic sample of I treated in the same way as the metabolite sample. The amount of unchanged drug in urine was very small, and quantitation was not practical. This finding indicated that only a trace amount of the unchanged drug was excreted in urine after intraperitoneal injection in rats.

The extracts from urine also showed a peak with *m/e* 129, at a column temperature of 120°, with a retention time equal to that of an authentic sample of III. The identity of this substance was further established by recording its mass spectrum between *m/e* 200 and 10. The mass spectrum obtained was virtually identical to that of an authentic sample of this compound. Compound III was present in a considerably larger amount than the unchanged drug.

The chromatographic separation of IV using the described conditions was not satisfactory. However, the separation of the silyl derivatives of III and IV was acceptable. Extracts from urine were silylated with *N,O*-bis(trimethylsilyl)acetamide to form the monosilyl derivative of III (mol. wt. 201) and the disilyl derivative of IV (mol. wt. 245). The silylated extract was examined by GLC–mass spectrometry using a 3% OV-1 column at 65° and monitoring *m/e* 201 (monosilyl derivative of III) and at 80° and monitoring *m/e* 245 (disilyl derivative of IV).

A peak of *m/e* 201 was observed, confirming the presence of III in urine. The quantitation of III in urine was achieved by comparing the absolute intensity of *m/e* 201 at the peak maximum with the intensities obtained by adding different known concentrations of the standard compound to rat urine and subjecting these samples to exactly the same processes as the experimental samples. Estimation of III in urine indicated that approximately 9% of the injected dose was excreted as this metabolite. No peak of *m/e* 245 (the disilylated derivative of IV) was detected in urine.

Since it was not possible to develop a GLC procedure for the detection and quantitation of II in urine, it was imperative to use an indirect method for its determination. This method involved the acid hydrolysis of urine and measurement of the increase in the amount of IV. Conjugated metabolites that may be excreted in urine after the intraperitoneal injection of I would produce III and IV on acid hydrolysis. Urine was subjected to acid hydrolysis and extracted in the same way as previously described. The GLC–mass spectrometric examination of these extracts directly and after silylation indicated the presence of only III. The amount of III in urine after acid hydrolysis was 11% of the injected dose, indicating that 2% of the injected dose was excreted in urine in the form of a conjugate of I or III and/or unchanged I. The absence of IV in the hydrolyzed urine indicated that neither II nor conjugates of II and IV were excreted in urine.

These findings are in agreement with those of Egli (7), who reported that approximately 9% of injected I was recovered in the 48-hr urine of rats as III and that 2% was recovered as conjugates that produced III on acid hydrolysis. Egli (7) was not able to detect the presence of the unchanged drug in urine.

However, these findings are in contradiction with the findings of Abood and Rinaldi (6), who reported that 96% of the injected dose of I was excreted unchanged in rat urine within 48 hr after intraperitoneal injection in rats. These investigators used tritium-labeled I with no provision to establish the position of the label in the molecule. The radioactivity they measured in rat urine may have been due to some metabolite not detected with the present analytical methods.

The absence of II and its hydrolysis product IV from urine was unexpected since II was a major metabolite of I *in vitro*. Also, it was disturbing that about 90% of the injected dose was not accounted for. Consequently, the metabolism of the potential metabolites, II–IV, was studied to determine whether they are terminal metabolites or may be further metabolized to other products.

Extracts from urine of rats injected with II hydrochloride contained the disilyl derivative of IV in an amount approximately 18% of the injected dose. The amount of IV in urine after hydrolysis was approximately the same as before hydrolysis, indicating that no unchanged II or a conjugate that produced IV on acid hydrolysis was present.

These findings indicated that hydrolysis was one metabolic pathway for II. However, the major route of elimination of this compound as well as I is not as yet known. The presence of IV in urine of rats injected with II and its absence in the urine of rats injected with I indicated that II is probably not a major metabolite of I.

Approximately 26% of the injected dose of III hydrochloride was present in urine as the unchanged drug. The amount of III in urine after acid hydrolysis was 89% of the injected dose, indicating that 63% of the injected dose was excreted in urine in the form of conjugates that produced III on acid hydrolysis. The extracts from urine did not contain the potential metabolite IV.

Extracts from urine of rats injected with IV hydrochloride contained approximately 50% of the injected dose as the unchanged drug. The amount of IV in urine after acid hydrolysis was approximately 100% of the injected dose, indicating that 50% of the injected dose was excreted in urine in the form of conjugates that produced IV on acid hydrolysis.

EXPERIMENTAL[2]

Compound I Hydrochloride—The title compound was obtained in 72% yield from III and methyl benzilate as described by Biel *et al.* (1). Recrystallization from absolute ethanol gave pure I hydrochloride, mp 190–192° [lit. (1) mp 191–192°].

Anal.—Calc. for $C_{21}H_{25}ClNO_3$: C, 67.11; H, 6.92; Cl, 9.45; N, 3.72. Found: C, 66.89; H, 7.16; Cl, 9.84; N, 3.54.

Compound II Hydrochloride—*N*-Benzyl-3-chloropiperidine (6.7 g, 30 mmoles) was dissolved in dry isopropyl alcohol (30 ml). To this solution was added benzilic acid (10.29 g, 45 mmoles) in dry isopropyl alcohol (90 ml), and the mixture was heated under reflux for 24 hr. Isopropyl alcohol was then removed by evaporation under reduced pressure. The precipitate was partitioned between 5% potassium carbonate aqueous solution and ether (60 ml). The ether layer was evaporated to dryness, and the residue was dissolved in a minimum volume of isopropyl alcohol; hydrogen chloride-saturated ether was added to precipitate the hydrochloride salt.

The resulting *N*-benzyl-3-piperidyl benzilate hydrochloride was filtered and recrystallized from absolute ethanol. It weighed 5.3 g (40.4% yield), mp 204.5° dec. *N*-Benzyl-3-piperidyl benzilate (4.6 mmoles) was dissolved in methanol (60 ml) containing acetic acid (0.4 ml), and 5% palladium-on-charcoal (900 mg) was added. The mixture was shaken under 46.5 psi of hydrogen for 90 min and filtered. The filtrate was concentrated to about one-third of its volume under reduced pressure. Hydrogen chloride-saturated ether (40 ml) was added and evaporated to dryness.

The residue was dissolved in methanol, and sufficient ether was added to effect complete precipitation. The precipitate was filtered, washed with ether, and dried *in vacuo* to provide 1.435 g (90% yield). Recrystallization from absolute ethanol gave 1.435 g of II hydrochloride (71% yield), mp 179.5–182°; NMR ($CDCl_3$): δ 7.25 (m, 10H), 4.8 (m, 1H), 3.4 (m, 2H), 2.5 (m, 4H), and 1.5 (m, 4H); mass spectrum: *m/e* (relative intensity), 312 (0.2), 212 (0.3), 183 (42.4), 105 (68.1), 84 (20.8), 83 (100), and 76 (1.7).

Compound III Hydrochloride—The hydrochloride was prepared by dissolving III[3] in methanol and adding hydrogen chloride-saturated ether. The precipitate was collected and recrystallized from isopropyl alcohol–ether, mp 159–162°; NMR (free base in $CDCl_3$): δ 3.0 (s, 1H), 3.6 (m, 1H), 1.1 (t, 3H, J = 7 Hz), 2.35 (q, 2H, J = 7 Hz), 2.35 (m, 4H), and

[2] Melting points were determined in open capillary tubes and are *uncorrected*. IR spectra were obtained with a Perkin-Elmer 237B spectrophotometer in potassium bromide disks. NMR spectra were obtained in deuterochloroform with a Varian A-60D spectrometer, using tetramethylsilane as the internal standard. Microanalyses were performed by M-H-W Laboratories, Garden City, Mich. Mass spectra were obtained with an AEI MS-30 mass spectrometer. GLC analyses were carried out on a Perkin-Elmer 900 instrument equipped with a flame-ionization detector. GLC–mass spectral analyses were performed on an LKB 9000 instrument equipped with a PDP 8E data processing system.

[3] Aldrich Chemical Co., Milwaukee, Wis.

1.68 (m, 4H); mass spectrum: *m/e* (relative intensity) 129 (17.39), 114 (100), 199 (6.77), 84 (9.16), 72 (32.71), 58 (14.91), 57 (40.37), 44 (21.74), 43 (20.91), 42 (58.18), and 41 (18.63).

Compound IV Hydrochloride—Compound IV[3] was dissolved in methanol and treated with hydrogen chloride-saturated ether until complete precipitation of the hydrochloride salt was observed. The precipitate was recrystallized from isopropyl alcohol–ether, mp 193–196°; NMR (free base in $CDCl_3$): δ 3.7 (m, H), 3.45 (s, H), 2.96 (m, H), 2.75 (m, 4H), and 1.65 (m, 4H); mass spectrum: *m/e* (relative intensity) 101 (42.61), 83 (2.20), 57 (65.25), 56 (35.85), 55 (12.74), 44 (100), 43 (32.23), 42 (24.06), and 41 (16.35).

***In Vitro* Studies**—*Tissue Preparations*—Livers from male Sprague–Dawley rats, 150–200 g, were used as the source of liver homogenates and microsomal preparations. Blood from the same rats was used for measurement of blood esterase activity. The animals were allowed food and water *ad libitum*. They were stunned by a blow on the head and decapitated, and the blood was collected in heparinized beakers.

The livers were removed rapidly and placed in ice-cold 1.15% KCl solution. All further preparations were conducted at 0–4°. The livers were mixed with three times their weight of 1.15% KCl and homogenized[4], and the homogenate was centrifuged in a refrigerated centrifuge[5] at 10,000×*g* for 30 min. The supernate was withdrawn, and half of it was used in studies with liver homogenates. The other half was centrifuged[6] at 105,000×*g* for 60 min. The high-speed supernatant layer was used as the "supernatant fraction of liver homogenate," and the precipitate, the microsomal pellet, was resuspended in 1.15% KCl solution.

Incubation Conditions—All solutions for enzyme preparation and incubation mixtures were prepared in water double distilled from glass. The incubation mixture contained 10 ml of 10 m*M* substrate (I, II, or III), 10 ml of enzyme preparation (liver homogenate, microsomal fraction, supernate of liver homogenate, or blood), 5 ml of cofactors solution (2.25 μmoles of NADP and 25 μmoles of glucose 6-phosphate in 1.15% KCl), 20 ml of buffer (prepared from 10 μmoles of nicotinamide, 5 μmoles of magnesium chloride, 1.63 g of dibasic sodium phosphate/100 ml, and 0.53 g of monobasic potassium phosphate/100 ml and adjusted to pH 7.4 with sodium hydroxide), and 5 ml of 1.15% KCl solution. The total volume was 50 ml.

Incubations were conducted at 37° in an open atmosphere for 2 hr in 250-ml conical flasks placed in a water bath shaker[7]. The reactions were terminated by the addition of 10 ml of 0.6 *N* perchloric acid. The incubation mixtures used in the zero-time studies were identical to the described mixtures without the enzyme preparation. Addition of the enzyme preparation was carried out in the last step, after the addition of perchloric acid. The mixtures were centrifuged, and the supernate was used for the identification of metabolites.

Identification of Metabolites—The supernate was extracted with an equal volume of benzene to remove lipids. The aqueous phase was adjusted to pH >10 with dilute ammonium hydroxide and extracted first with methylene chloride (75 ml) and then with 75 ml of chloroform–isopropyl alcohol (3:1). The extracts were combined and evaporated to dryness in a nitrogen stream, and the residues were dissolved in 0.1-ml portions of methylene chloride.

The residues obtained from each incubation were applied onto 10 silica gel GF TLC plates[8] (20 × 20 cm, 250 μm). The plates were developed in acetonitrile–chloroform–methanol–ammonium hydroxide (50:40:29:1) in one dimension and then in ethyl acetate–methanol–ammonium hydroxide (100:100:1) in a second dimension. The dry plates were examined under a UV lamp for the detection of I and II, and one plate was sprayed with 0.5% $KMnO_4$ to locate the metabolites. Areas of the plates corresponding to the positions of the authentic samples were scraped off the remaining nine plates.

The adsorbent fractions obtained from the same area on different plates were combined and extracted with methanol. The methanol extracts were dried in a nitrogen stream, and the residues were submitted for mass spectral analysis. The residue obtained from the area on the TLC plates corresponding in position to that of an authentic sample of II was dissolved in 0.1 ml of chloroform and mixed with 200 mg of potassium bromide (IR grade). The dried mixture was used for the preparation of a pellet for IR spectroscopy.

The residues obtained by evaporation of the extracts from the incubation mixtures (before application to the TLC plates) also were examined by GLC–mass spectrometry, using a 12% Carbowax 20M column at 120° to detect metabolites III and IV. The mass spectrum of the peak eluted with the same retention time as authentic III was recorded. No chromatographic peaks with the same retention times as authentic IV were observed in any extract.

***In Vivo* Studies**—Three male Sprague–Dawley rats, 500 g, were used for each experiment. The rats were placed individually in metabolism cages and allowed food and water *ad libitum;* urine (control) was collected for 24 hr. The rats were injected intraperitoneally with solutions of the hydrochloride salts of I, II, III, or IV in normal saline at 80 mg/kg in four equal injections for I, III, and IV or at 60 mg/kg in five equal injections for II. The urine was collected under toluene for 72 hr after injection. Urine samples were removed from the metabolism cage periodically and stored at −25° until analyzed.

Preparation of Correlation Curves—A 30-, 20-, 10-, or 7.5-mg portion of authentic III hydrochloride was added to separate 10-ml aliquots of control urine. The solutions were made alkaline (pH 8.0) by the addition of dilute ammonium hydroxide. A 20-ml aliquot of 0.2 *M* carbonate buffer (pH 11) and 5 ml of the basic charcoal suspension were added to each urine sample. The basic charcoal suspension was prepared by adding 15 g of charcoal[9] to 400 ml of carbonate buffer (sodium carbonate–sodium bicarbonate, pH 11) and mixing thoroughly, allowing the charcoal to settle overnight, and then aspirating as much as possible of the supernatant buffer.

The mixtures were mixed thoroughly by repeated gentle shaking for 30 min and then centrifuged at 4000×*g* for 15 min. The clear supernatant layers were discarded, and the charcoal precipitates were shaken with 150 ml of methanol. The methanol extracts were filtered and evaporated to dryness in a nitrogen stream. The residues were each treated with 235 mg of *N,O*-bis(trimethylsilyl)acetamide to form the monosilyl derivative of III. The mixtures were examined by computer-equipped GLC–mass spectrometry on 3% OV-1 at 65°, using the mass spectrometer as a specific ion detector. A correlation curve was obtained by plotting the concentration of authentic III *versus* the absolute intensity of the ions of *m/e* 201 at the peak maximum for the monosilyl derivative of III. Regression analysis[10] was used to determine the line of the best fit for the data points.

A 7.5-mg portion of authentic IV hydrochloride was added to a 10-ml aliquot of urine. The standard sample was then extracted as described. The residue after silylation was examined by computer-equipped GLC–mass spectrometry using a 3% OV-1 column at 80°.

Examination of Urine for I, III, and IV—An aliquot of urine (one-third to one-fourth the urine sample) was adjusted to pH 11.0 and treated with a suspension of charcoal, and the charcoal was extracted with methanol as previously described. The methanol extract was evaporated in a nitrogen stream, and the residue was dissolved in methylene chloride. The detection of I in urine was achieved by examining the solution by computer-equipped GLC–mass spectrometry using a 3% OV-1 column at 220°. The mass spectrometer was used in the specific ion detector mode to monitor the peaks with *m/e* 339.

The methylene chloride solution was evaporated to dryness in a nitrogen stream, and the residue was treated with 609 mg of *N,O*-bis(trimethylsilyl)acetamide. The solution was examined again by computer-equipped GLC–mass spectrometry using a 3% OV-1 column at 65° and monitoring the ions with *m/e* 201 for the monosilyl derivative of III. The column temperature was raised to 80°, and the sample was reinjected; the mass spectrometer was set to monitor ions with *m/e* 245 for the detection and quantitation of the disilyl derivative of IV.

Examination of Urine for Compounds that Produce III or IV after Acid Hydrolysis—An aliquot of the urine sample was mixed with an equal volume of 12 *N* HCl and heated at 118° for 16 hr. The mixture was adjusted to pH 11.0 and treated with a suspension of charcoal, and the charcoal was extracted with methanol as previously described. The methanol extract was evaporated in a nitrogen stream, and the residue was treated with 609 mg of *N,O*-bis(trimethylsilyl)acetamide. The solution was examined for the presence of the monosilyl derivative of III and the disilyl derivative of IV by computer-equipped GLC–mass spectrometry as previously described.

REFERENCES

(1) J. H. Biel, L. G. Abood, W. K. Hoya, H. H. Leiser, P. A. Nuhfer, and E. Kluchesky, *J. Org. Chem.*, **26**, 4096 (1961).

[4] Polytron ultrasonic homogenizer, Brinkmann Instruments, Westbury, N.Y.
[5] Beckman J-21, Beckman Instruments, Fullerton, Calif.
[6] Beckman L2-65B ultracentrifuge, Beckman Instruments, Fullerton, Calif.
[7] Eberbach Corp., Ann Arbor, Mich.
[8] Analtech, Newark, Del.
[9] Merck and Co.
[10] Hewlett-Packard 9100A calculator.

(2) L. G. Abood and J. H. Biel, *Int. Rev. Neurobiol.*, **4**, 218 (1962).
(3) L. G. Abood, A. Astfeld, and J. H. Biel, *Arch. Int. Pharmacodyn. Ther.*, **120**, 186 (1959).
(4) L. G. Abood and L. J. Meduna, *J. Nerv. Ment. Dis.*, **127**, 546 (1958).
(5) D. C. English, *J. Neuropsychiat.*, **3**, 304 (1962).
(6) L. G. Abood and F. Rinaldi, *Psychopharmacologia*, **1**, 117 (1959).
(7) K. Egli, Master's thesis, University of Minnesota, Minneapolis, Minn., 1973.
(8) M. W. Anders and G. J. Mannering, *Mol. Pharmacol.*, **2**, 219 (1966).
(9) L. F. Zerilli, A. Cometti, N. Rimorini, and G. G. Gallo, in "Proceedings of the International Symposium on Gas Chromatography Mass Spectrometry," A. Frigerio, Ed., Elba, Italy, 1972.
(10) J. M. Meola and M. Vanko, *Clin. Chem.*, **20**, 184 (1974).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 20, 1976, from the *College of Pharmacy and the ‡College of Biological Sciences, University of Minnesota, Minneapolis, MN 55455.

Accepted for publication August 18, 1976.

Supported in part by a grant from the research funds provided by the Graduate School of the University of Minnesota.

The authors also thank the Samuel W. Melendy Summer Fellowship for financial support to C.-H. Chen.

ˣ To whom inquiries should be directed.

Effects of Agitation on Size Distribution of Particulate Matter in Large-Volume Parenterals

JAMES BLANCHARD ˣ, JOHN A. SCHWARTZ, and DALE M. BYRNE *

Abstract □ The particle-size distributions of six types of large-volume parenterals subjected to different degrees of agitation were determined using an automatic particle counter. Data acquired from each solution, which had been maintained in a stored condition, subjected to agitation by inverting 20 times, and then mechanically shaken for 30 min, produced a linear relationship between log $N_{>D}$ and log D. Both the slope (K) and the number of particles per milliliter exceeding 1 μm in diameter ($N_{>1}$) exhibited a dependence on the degree of agitation. Their combined effect indicates that agitation by 20 hand inversions removed particulate matter from the surface of the container, which increased the total number of particles in solution (>1 μm) but did not significantly alter the relative size distribution. Agitation for 30 min, however, disintegrated agglomerates and produced a particle-size distribution with a greatly increased number of particles whose diameters were less than 1 μm and a corresponding decrease in the number of particles exceeding 1 μm in diameter. The particle-size distribution of a parenteral solution determined by this *in situ* instrumental method was, therefore, dependent upon the degree of agitation to which the parenteral was subjected prior to examination.

Keyphrases □ Particle-size distribution—various large-volume parenterals, effect of different degrees of agitation □ Parenterals, large volume—particle-size distribution, effect of different degrees of agitation □ Dosage forms—various large-volume parenterals, particle-size distribution, effect of different degrees of agitation □ Agitation—effect on particle-size distribution of various large-volume parenterals

Particulate matter was defined as "extraneous, mobile, undissolved substances, other than gas bubbles, unintentionally present in parenteral solutions" (1). In a previous report, efforts to compare and contrast the ability of five methods to monitor the levels of particulate matter were described (2). During this investigation, it became apparent that the automatic particle counter was suitable for the rapid determination and sizing of particulate matter. This instrument's ability to provide a reasonably accurate determination of the particle-size distributions present in large-volume parenterals was comparable[1] to the membrane filtration and microscopic examination technique utilized in the recently adopted USP–NF standard (1).

Some difficulties previously were noted in determining the actual levels of particulate matter of a given size present in parenterals. Ernerot *et al.* (3), who utilized a destructive counting procedure, reported that the particle-size distribution in a parenteral solution at a given time was largely dependent upon the degree of agitation the solution had previously received. By its very nature, a destructive counting technique introduces a certain amount of shear force upon the particles in the solution, thereby altering the initial distribution of particles. This shear force introduced by the measuring technique itself would be eliminated by a nondestructive technique, since it would not be necessary to remove the solution from its container. Therefore, a nondestructive *in situ* technique that does not impose any additional shear force on the particles in a solution would be ideally suited for characterizing the effects of agitation on particle-size distributions in parenteral solutions.

Some recent reports gave conflicting accounts of the importance of the effects of agitation. Groves (4) attributed the increase in particle counts observed (3) to the presence of air bubbles generated during the agitation procedure. Ernerot (5) refuted this argument and identified the disintegration and flocculation of particles as the true sources of the variation in particle counts following agitation. Blaug and Sarabia (6) noted that the particle counts recorded using an electronic particle counter were the same for samples taken from intravenous bottles at rest or from the same bottles shaken immediately prior to sampling. Since destructive particle counting techniques were used in these investigations, it is difficult to draw definitive conclusions regarding the actual effects of agitation. Therefore, a major goal of this study was to examine more definitively the effects of agitation on the particle-size distribution in large-volume parenterals utilizing a nondestructive instrumental technique.

[1] J. Blanchard, J. A. Schwartz, and D. M. Byrne, to be published.