methylated derivatives, which in the case of estradiol was identified as 2-methoxyestradiol by paper chromatography.

Simple phenols, naphthols, and hydroxyquinolines undergo hydroxylation to catechols. This reaction has also been demonstrated previously *in vivo* for various simple phenols and hydroxyqinolines.[26] In our studies both 4- and 8-hydroxyquinoline are hydroxylated while the 2-hydroxy isomer which exists mainly in the 2-keto form does not react. Various dimethylphenols are active except for 2,6-dimethylphenol in which catechol formation would require replacement of a methyl group by hydroxyl. Similarly various dichlorophenols were active, and even 2,4,6-trichlorophenol was converted to a methylated product although both *ortho* positions are blocked by chloro groups. Since NADP and glucose-6-phosphate are required, direct methylation of the phenol without an oxidative step is unlikely. Also catechol-O-methyltransferase involvement is indicated since the formation of the O-methylated derivative requires magnesium ions and is inhibited by tropolone.[12] Direct O-methylation by the enzyme which O-methylates 2,6-diiodophenols, described by Tomita, *et al.*,[27] does not seem likely since their enzyme does not require magnesium ions. It seems more likely that a replacement of a chloro group by a hydroxyl group, similar to that described by Kaufman[28] for the conversion of *p*-chloro- or *p*-fluorophenylalanine to tyrosine, has occurred. Certain other 2,6-dihalophenols also show low activity in our microsomal system.

Little correlation of activity with structure was possible for the various monosubstituted phenols (Table VI) studied. Since catechol-O-methyltransferase is present in a large excess and is little influenced by substituents[6] so that O-methylation is observed even in the presence of very bulky *ortho* substituents such as *t*-butyl,[29] most of the effects observed are probably related to hydroxylation. Both *para*- and *meta*-substituted compounds were quite active with *ortho* substitution, often severely reducing activity especially with very bulky groups such as *t*-butyl or with groups that can interact by hydrogen bonding with the phenol such as in salicylaldehyde. Compounds sensitive to oxidation such as aminophenols showed low activity, perhaps due to competitive oxidations.

(26) R. I. Williams, "Biochemistry of Phenolic Compounds," J. B. Harborne, Ed., Academic Press Inc., London, 1964, Chapter 6.

(27) K. Tomita, C. M. Cha, and H. A. Lardy, *J. Biol. Chem.*, **239**, 1202 (1964).

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Excretion, Distribution, and Metabolism of Doxapram Hydrochloride

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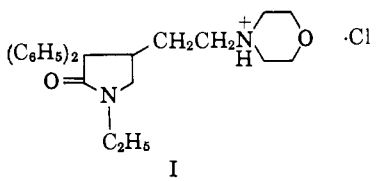
Received August 24, 1964

Doxapram hydrochloride, following intravenous injection into the dog, was rapidly metabolized. No unchanged drug was found in any biological material analyzed. The metabolites formed were distributed throughout the animal body with higher levels occurring in the fat, liver, pancreas, and adrenal glands than in other tissues. Blood levels of the metabolites decreased rapidly during the first hour followed by a much more gradual decline. Large concentrations of the metabolites occurred in the bile. The metabolites were excreted rapidly in the urine during the first 24 hr. Small amounts continued to be excreted up to 120 hr. after injection of doxapram hydrochloride. The primary path of metabolism was through opening of the morpholine ring. A large number of metabolites were formed, two of which were identified as 1-ethyl-4-(2-hydroxyethylaminoethyl)-3,3-diphenyl-2-pyrrolidinone and 1-ethyl-4-(2-aminoethyl)-3,3-diphenyl-2-pyrrolidinone.

Doxapram hydrochloride[1] is the nonproprietary name for 1-ethyl-4-(2-morpholinoethyl)-3,3-diphenyl-2-pyrrolidinone hydrochloride (I). This compound is



I

an agent that produces marked respiratory stimulation and pressor effects in animals[2a,b] and in humans.[2c,d] When doxapram is administered to animals under barbiturate-induced sleep, it produces arousal effects.[2a,c]

In this paper we present the results that have been obtained in studies designed to elucidate the metabolism, distribution, and excretion of doxapram. To carry out such studies it has been necessary to develop analytical methods suitable for the determination of the drug in the various biological materials. The methods are ultimately based on the oxidation of doxapram and doxapram-like materials to benzophenone which is subsequently quantitated from its ultraviolet absorption. The methods described, therefore, will determine any material which contains the diphenylmethyl moiety and which appears in the material finally oxidized.

Correlation of the results obtained from the "oxidation method" with those found using C^{14}-labeled doxapram indicate that all of the metabolites formed may be determined by the choice of the appropriate procedure for the "oxidation method."

(1) C. D. Lunsford, A. D. Cale, Jr., J. W. Ward, B. V. Franko, and H. Jenkins, *J. Med. Chem.*, **7**, 302 (1964).

(2) (a) J. W. Ward and B. V. Franko, *Federation Proc.*, **21**, 325 (1962); (b) W. H. Funderburk and R. S. Alphin, *ibid.*, **21**, 324 (1962); (c) A. J. Wasserman and D. W. Richardson, *Clin. Pharmacol. Therap.*, **4**, 321 (1963); (d) H. G. Canter, *Am. Rev. Respirat. Diseases*, **87**, 830 (1963).

Experimental

Analytical Methods.—Initially, the ultraviolet spectrum of doxapram was considered as a method for quantitation. The

absorption coefficient proved, however, to be too low to determine the small amounts that might be present in biological materials. This spectrum showed four distinct maxima at 269.5, 264.5, 258.5, and 253 mμ. Compounds chemically related to doxapram, *i.e.*, those containing the diphenylpyrrolidinone moiety, gave similar ultraviolet spectra. In some cases the maxima were displaced. The spectra have proved of great value in the separation and identification of metabolites.

The ultraviolet absorption of benzophenone, in contrast to that of doxapram, is very strong. The oxidation of doxapram, under proper conditions, should yield this ketone. Attempts were made to oxidize with chromic acid, but the results were not reproducible. Alkaline permanganate oxidation in a boiling-water bath gave consistent results, and the quantity of doxapram added was directly proportional to the benzophenone absorption found.

It was found possible to separate benzophenone from the cooled oxidation solution by extraction with isooctane. This solvent would not completely extract the benzophenone from the alkaline oxidation mixture. However, if the mixture were first made acidic with phosphoric acid, extraction appeared to be complete. Other acids, sulfuric or hydrochloric, appeared to be oxidized under these conditions and resulted in ultraviolet absorption which completely masked the benzophenone spectrum.

Some samples could be oxidized directly by the method described. This was true when the concentration of the benzophenone-yielding material was sufficiently high, as in the case of urine or bile. The limiting factor was the capacity of the potassium permanganate added. Because of this, organic solvents used for extraction must be removed completely prior to the oxidation. Tissues were extracted with benzene or ether, and the solvent was removed from the extract before oxidation was carried out. The detailed procedures used in these investigations are as described below.

Bile and Urine.—An aliquot of bile (0.10 ml.) or urine (0.10–0.50 ml.) was transferred to a 200 × 16 mm. test tube having a 24/40 ⅁ joint (oxidation tube). To this sample 0.5 ml. of 2 *N* NaOH and 5 ml. of 5% $KMnO_4$ were added. An air condenser (250 × 6 mm.) was connected to the test tube and the sample was heated for 1 hr. in a boiling-water bath. The sample was cooled and made acidic by adding 1 ml. of 6 *N* H_3PO_4 through the air condenser. The condenser was removed and 2.0 ml. of Spectro Grade isooctane was added immediately. The contents of the tube were mixed for 1 min. on a Vortex mixer to extract the benzophenone into the isooctane. The isooctane layer was transferred to a microcuvette (1-cm. path length), and its spectrum was determined from 230–260 mμ. A base line was drawn from 237 to 256 mμ and the peak height was measured from this line to the maximum using a millimeter ruler. This length was converted to concentration by a predetermined factor or curve prepared from pure doxapram under the same conditions.

Blood and Cerebrospinal Fluid.—A sample of 5.0 ml., or less, of blood or cerebrospinal fluid was transferred to a 50-ml., glass-stoppered centrifuge tube containing 20 ml. of water, 1.0 ml. of 5 *N* NaOH, and 10.0 ml. of benzene. The tube was shaken gently for 5 min. Emulsions were broken by centrifugation. A 5-ml. aliquot of the clear benzene layer was transferred to an oxidation tube, and the benzene was evaporated in a boiling-water bath. To ensure complete removal of the benzene, 2–3 ml. of absolute ethanol was added after the contents of the tube appeared dry, and the ethanol was evaporated in boiling water with the aid of a stream of air. Potassium permanganate and alkali were then added and the procedure was carried out as described above for urine and bile.

Other Tissues.—Samples of other tissues, in quantities up to 5 g., were ground with sufficient anhydrous Na_2SO_4 so that they appeared dry. The mixture was transferred to a thimble and extracted in a Soxhlet apparatus for 4 hr. Nervous tissue, *i.e.*, brain or spinal cord, was extracted with ethyl ether; other tissues were extracted with benzene. The precipitate obtained on cooling the ether extracts was separated by centrifugation and decantation. The extract (benzene or ether) was transferred to a separatory funnel and the doxapram was extracted with two 10-ml. portions and then 5 ml. of 2 *N* HCl. The HCl extracts were combined in another separatory funnel and made alkaline with 6 ml. of 10 *N* NaOH. The alkaline solution was extracted three times with 5-ml. portions of $CHCl_3$. The lower layers were dried through anhydrous Na_2SO_4 and combined in an oxidation tube. The solvent was evaporated, as described above for benzene, and oxidation was carried out.

Steam Distillation Procedure.—Attempts to analyze some samples, particularly feces, by the above procedures yielded variable and inconsistent results. A procedure was therefore devised to separate the benzophenone from the oxidized sample by steam distillation. It was not necessary to prepare extracts of samples when this variation of the procedure was used.

A sample of tissue (25–35 g.) was weighed accurately and transferred to an erlenmeyer flask. The tissue was dissolved by adding 50 ml. of 2 *N* NaOH and heating on the steam bath for 1 hr. with frequent swirling. The solution was cooled and an aliquot containing 2.0 g. of tissue (0.5 g. of fat) was transferred to a 300-ml. Kjeldahl flask having a 24/40 standard taper joint. Sixty milliliters of a saturated solution of potassium permanganate and sufficient NaOH to make the solution 0.2 *N* were added. An air condenser, as described previously, was attached, and the flask was heated in a boiling water bath for 1 hr. with frequent shaking. The flask was cooled and attached to an all-glass steam distillation apparatus. The benzophenone was steam distilled into a 100-ml. glass-stoppered graduate containing 2.0 ml. of isooctane, so that the distillate was delivered below the surface of the isooctane. The distillate, after collection of 100 ml., was made acidic and shaken thoroughly to transfer the benzophenone to the isooctane. The ultraviolet spectrum was then determined and analyzed, as previously described.

Brain tended to foam over during this procedure; however, this was easily prevented by adding 20 g. of solid $BaCl_2$ to the oxidation mixture prior to steam distillation.

Chromatography.—Thin layer chromatography was carried out using inactivated silica gel plates prepared in the usual manner. A number of different solvent systems were used. However, methanol or ethanol proved to be satisfactory mobile solvents in most cases. Spots were located by spraying with aqueous $KMnO_4$ or by irradiation. Following irradiation of the developed plate for 15–30 min. with short wave-length ultraviolet light (2537 Å.), compounds containing the diphenylpyrrolidinone moiety became fluorescent and could be seen under the longer wave-length (3660 Å.) light.

Isotope Studies.—Cyano-C^{14}-diphenylmethane was prepared from $KC^{14}N$ by the method as described by Sisido, *et al.*[3] From this compound, 1-ethyl-4-(2-morpholinoethyl)-3,3-diphenyl-2-pyrrolidinone-2-C^{14} was synthesized by the procedure described by Lunsford, *et al.*[4] The over-all yield was 13.0% and the specific activity was 0.74 and 0.16 μc./mg. in each of two fractions obtained by crystallization. The melting points agreed with that of doxapram hydrochloride and thin layer chromatography indicated only one radioactive and permanganate-positive material.

Biological samples were counted using a Packard liquid scintillation spectrometer, Series 314E, in glass vials. The aqueous phosphor consisted of toluene, dioxane, and ethyl alcohol (in the ratio of 4:4:2.4) containing 80 g. of naphthalene and 5 g. of PPO/l. The nonaqueous phosphor was 4 g. of PPO/l. of toluene.

Blood or spinal fluid (0.20 ml.) was introduced into 10 ml. of aqueous phosphor in a LSC vial through a 25-gauge needle from a syringe. The vial was shaken for 30 min. and centrifuged.[5] The sample was counted and corrected for quenching with benzoic acid-C^{14} as an internal standard. Bile and urine were counted by mixing 0.10 ml. with 10 ml. of aqueous phosphor.

Tissues and feces were counted after oxidation to CO_2. They were dried in a vacuum oven at approximately 55°, and ground in a blender; or, where only small amounts of tissue were available, the tissue was homogenized first and an aliquot was dried in a combustion boat. The dried sample was combusted in a combustion furnace by a modification of the method as described by Peets, *et al.*[5] The liberated $C^{14}O_2$ was counted in the nonaqueous phosphor.

Excretion Studies.—Mongrel dogs of either sex were used. A single dose of doxapram hydrochloride in water was administered intravenously. During the study the regular laboratory diet was given. Urine, feces, and blood samples were taken at intervals for analysis.

Tissue Studies.—Mongrel dogs, following an overnight fast, were anesthetized i.v. with phenobarbital sodium (125 mg./kg.). Carotid arterial blood pressure and respiration were recorded oscillographically, the ureters and common bile duct (cystic duct was ligated) were cannulated for collection of urine and bile.

(3) K. Sisido, H. Nozaki, M. Nozaki, and K. Okano, *J. Org. Chem.*, **19**, 1699 (1954).

(4) J. F. Nash and R. E. Crabtree, *J. Pharm. Sci.*, **50**, 134 (1961).

(5) E. A. Peets, J. R. Florini, and D. A. Buyske, *Anal. Chem.*, **32**, 1465 (1960).

Cerebrospinal fluid (CSF) was obtained by direct puncture into the cisterna magna, and blood was taken from a femoral vein. Saline was infused (3 ml./min.) so as to insure a good urine flow. Samples of body fluids were taken prior to and following the i.v. injection of 20 mg./kg. of doxapram hydrochloride. The animals were sacrificed by exsanguination, and samples of the following tissues were taken: heart, lung, liver, pancreas, kidney, adrenal, spleen, small intestine, large intestine, fat, skeletal muscle, spinal cord, cortex, medulla, and the remainder of the brain.

Results and Discussion

Analytical Methods.—The results from the recovery of doxapram, when known amounts were added to samples of various biological materials, is shown in Table I. This table also shows standard deviations obtained from representative tissues. The results of recoveries of fat and brain by the extraction method were somewhat low, probably due to difficulties encountered in the extraction procedures.

Emulsions and precipitates were formed when benzene was used for the tissue extraction. Isooctane was used for the extraction of benzophenone from the oxidation media because other solvents that were immiscible with water and transparent in this ultraviolet region were oxidized to some extent by the permanganate. The use of the "base-line" method for measuring the absorbance was necessary. Control tissues, *i.e.*, tissues having no benzophenone-yielding material present, gave, on occasion, absorbing material. The ultraviolet curve of such material did not show a maximum in the region of 230–260 mμ, but showed a straight line. The measurement of the absorbance between the base line and the maximum absorbance for benzophenone in millimeters was purely a matter of convenience in this laboratory.

The steam distillation procedure offered a number of advantages over the extraction method. The latter method resulted in the determination of only basic materials, whereas all benzophenone-yielding materials were determined by the steam distillation procedure. In addition, there was less occasion to lose materials in transferring from one vessel to another. The recoveries obtained by this method are also shown in Table I and appear to be satisfactory for the determination of total doxapram and its metabolites in tissues.

Urinary Excretion.—The results obtained from the study of urinary excretion of doxapram-related materials are shown in Figure 1. This figure gives the average data and variation obtained from the study of 15 dogs by the benzophenone method. Also shown are data found in two dogs whose excretion was studied through the use of the isotope method.

These results indicate that the major portion of the urinary excretion occurs within the first 24 hr. following dosage. An average of approximately 33% of the dose was excreted within this time. In the 48-hr. period shown, approximately 40% of the dose was excreted. The data obtained from one dog using the radioactive compound are shown in the lower curve of this figure. This shows that smaller amounts of doxapram materials were determined as compared with the benzophenone method. This might indicate that the pyrrolidinone ring had opened with the loss of the labeled carbon. However, a second dog studied for a 4-hr. period, as shown in the upper curve, would not indicate this. These data are probably within the experimental errors of the two methods of analysis and are not believed to indicate any significant difference. The excretion in several dogs, including the one that received radioactive doxapram, were studied for 120 hr. The increase in the amount excreted in the 48–120-hr. period accounted for less than 2% of the dose. In all cases there was some continued excretion during this period.

The greatest amount found to be excreted in the urine was 48% of the administered dose. It was, therefore, apparent that the drug was being excreted through some other mechanism or being converted to a form that was not detected by our analytical methods.

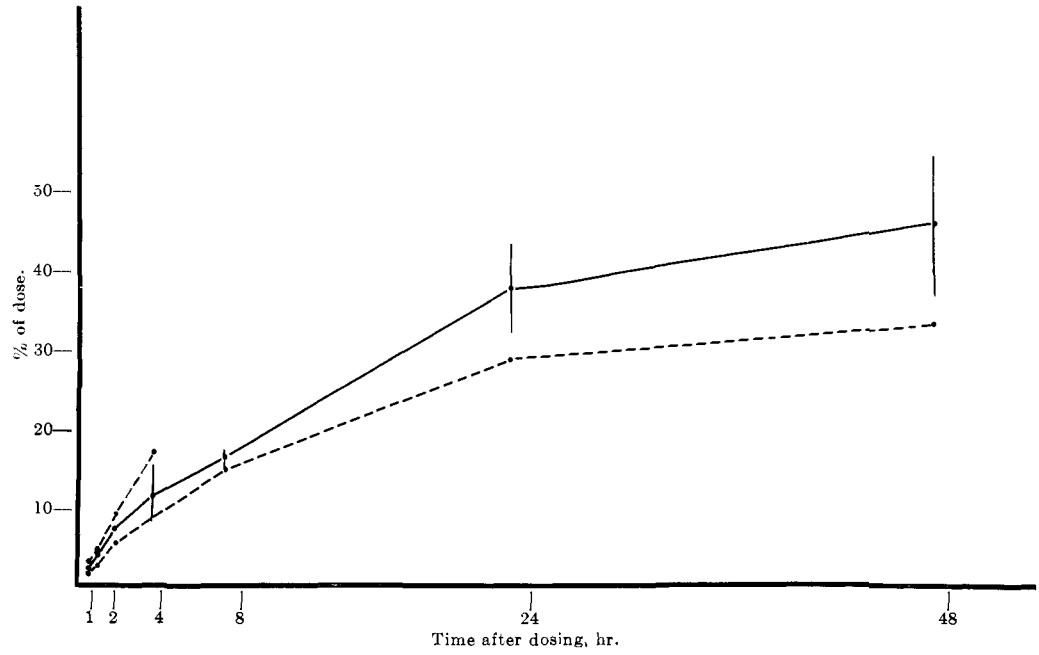


Figure 1.—Urinary excretion of doxapram-related materials in 15 dogs. The solid line shows the results obtained by chemical analysis and the broken line those obtained with radioisotopes. Vertical lines indicate the variation in results of the chemical analyses.

TABLE I

RECOVERY OF DOXAPRAM WHEN ADDED TO BIOLOGICAL MATERIAL

	Doxapram hydrochloride			
		Extraction method		Distillation method
Tissue	γ/g. (or ml.) added	No. of detns.	γ/g. found (S.D.)	γ/g. found
Blood	5.5	10	5.3 (±0.2)	
	10.0			9.0
	20.0			16.4
	25.2	2	22.2, 25.2	
	50.0	2	50.5, 55.0	
Fat	10.0			11.9
	20.0			20.0
	30.0			29.8
	50.0	10	42.0 (±1.9)	47.7
	100.0	2	76.0, 82.5	
	200.0	2	185, 182	
Muscle	6.3	6	5.7 (±0.3)	
	10.0	1	9.7	8.7
	20.0	1	22.3	18.0
	50.0			46.2
Urine	5.5	1	6.0	
	25.2	1	26.8	
	50.2	1	50.7	
	60.0	8	59.9 (±2.5)	
Bile	50.0	1	56.6	
	100.0	1	104.3	
	200.0	1	208.6	
Liver	5.0	1	5.2	
	10.0	1	11.2	9.7
	20.0	1	26.8	19.4
	30.0			27.6
Cortex	5.0	2	3.0, 6.0	
	10.0	2	6.7, 11.2	9.7
	20.0			20.8
Feces	20.0			22.3
	50.0			48.0

Fecal Excretion.—The fecal excretion was not studied as extensively as was the urinary excretion. The chemical method as first used was not satisfactory. Extraction methods removed only part of the total doxapram-related materials present. The results thus obtained were variable even between identical samples. Dog no. 180-60, which received labeled doxapram, was studied in this respect. The results of this study are shown in Table II. The major portion of that part excreted in the feces appeared in the first 24 hr. Some radioactivity continued to appear through 94 hr. following administration. In the feces of this dog 38% was found. Since urinary excretion accounted for 31%, the total recovered was 69% of the administered dose.

TABLE II[a]

FECAL EXCRETION OF DOXAPRAM-C^{14}-RELATED MATERIALS

Time after administration, hr.	Total doxapram-related materials excreted, mg.	% of C^{14} recovered
0–23.5	48.8	28.7
23.5–46.5	10.1	6.0
46.5–70.5	4.8	2.8
70.5–94.0	0.8	0.5
94.0–120.0	0	0

[a] This table shows the fecal excretion from a dog that was administered a single 20-mg./kg. i.v. dose of doxapram-C^{14}.

Blood Levels.—The results from the analysis of blood samples taken at various time intervals following administration are shown in Figure 2. As would be expected, the initial samples of blood showed the highest concentrations. The concentrations dropped rapidly during the first hour and then decreased more slowly through 24 hr. The concentrations at this time were extremely low. A comparison of the results obtained by the benzophenone method with those obtained by the isotope method shows that the latter are considerably higher.

The larger concentrations found in the isotope method leads one to suspect that a metabolite of doxapram exists in the blood stream that is not detected by the chemical method. Since there was no increase in the amount found by the steam distillation over that determined by extraction, the difference could not be due to the presence of an acidic compound. The possibility exists that hydroxylation of the phenyl group or groups may have occurred. Such compounds do not yield benzophenone under the conditions of the analysis. Such material should occur in the urine, which should be reflected in a difference between the isotope and chemical methods. As seen above, no such difference appeared to occur, unless it was too small to detect. The reason for the difference found in blood levels by the two methods is to be further investigated.

Bile Levels.—Bile, from the bile duct, was collected at intervals during the study of dogs used for tissue analysis. The results from the analyses are shown in Figure 3. Along with results from the chemical analyses is given the results obtained from one dog using the isotope technique. This curve falls in line with the results obtained by the chemical method. It was apparent that a considerable amount of doxapram materials appeared in the bile. The concentration in some of these samples was over 100 mg./100 ml. Initial samples, *i.e.*, at 5 min. following administration, did not contain detectable amounts of doxapram.

Tissue Analyses.—Results from the analyses of tissues from eight dogs administered doxapram intravenously at 20 mg./kg. are given in Table III. One dog, no. 341-62, was given isotopically labeled doxapram. The results from the analyses of the tissues of this dog were similar to the results obtained by the chemical method.

A study of the averages of the results indicates that doxapram-like materials are generally distributed throughout the animal. Higher levels are seen to occur in the fat, liver, pancreas, and adrenal glands. Nervous tissue, in general, contained somewhat less than other tissues. Abdominal and subcutaneous fat was analyzed separately in several dogs, but no significant differences in concentrations were obtained. The relatively large amounts of blood in liver and pancreas could account for the higher values found in these tissues.

Cerebrospinal Fluid.—Concentrations of doxapram or its metabolites were extremely low in cerebrospinal fluid. The highest concentration found was approximately 1.5 mg./100 ml., and most were less than 0.5 mg./100 ml. Since it was difficult to obtain samples of greater than 1–2 ml., the accuracy of such determinations is questionable. The spinal fluid of dog no.

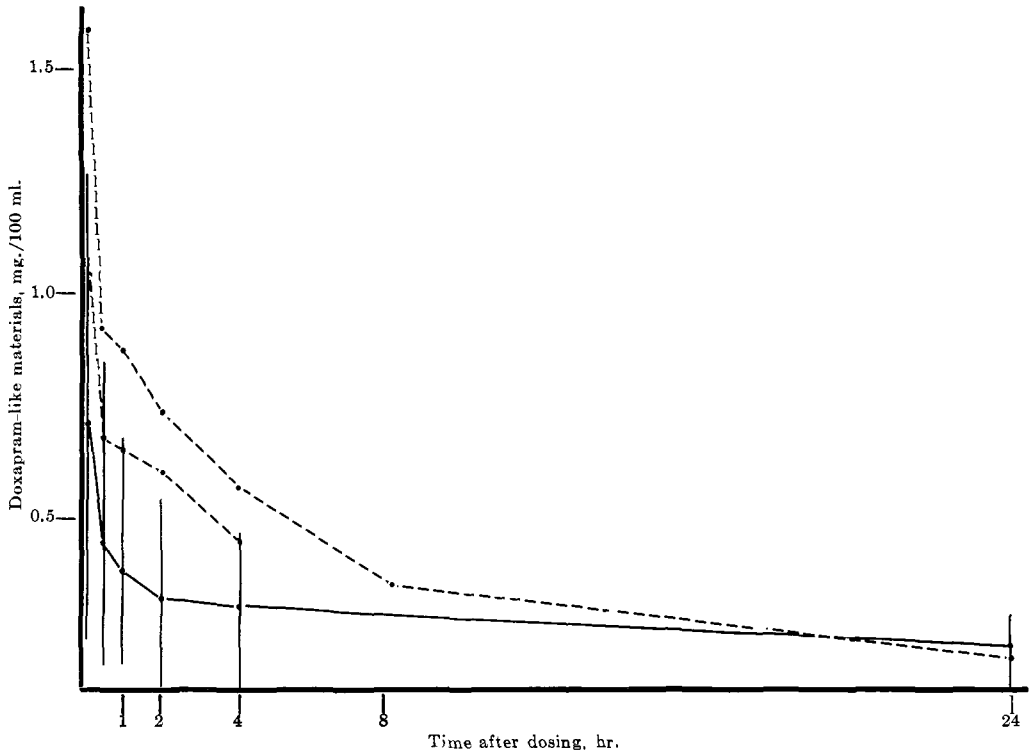


Figure 2.—Blood levels of doxapram-related materials in 12 dogs. The solid line shows the results obtained by chemical analysis and the broken lines those obtained with radioisotopes. Vertical lines indicate the variation in results of the chemical analyses.

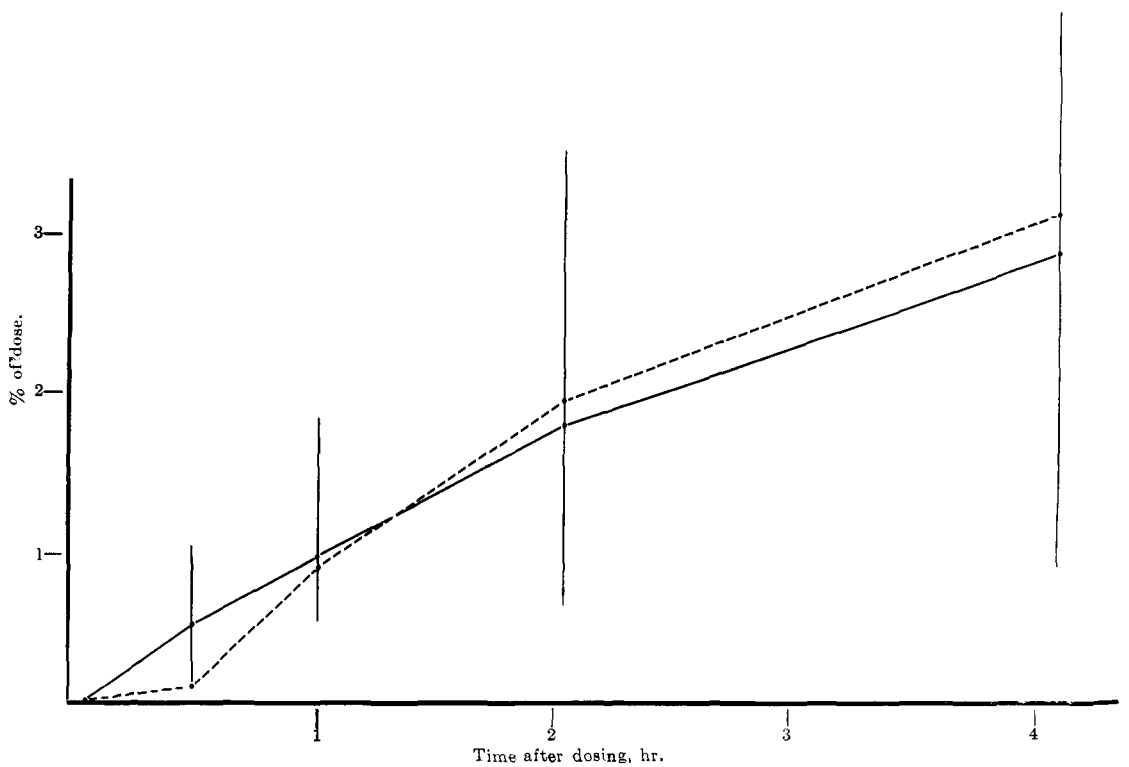


Figure 3.—Bile levels of doxapram-related materials in 8 dogs. The solid line shows the results obtained by chemical analysis and the broken line those obtained with radioisotopes. Vertical lines indicate the variation in results of the chemical analyses.

341-62, which received labeled doxapram, was analyzed 4 hr. following administration and found to contain 0.07 mg./100 ml., confirming that low concentrations were present.

Metabolites.—A number of attempts were made to identify unchanged doxapram in urine and in tissues. Urine, from dogs that received i.v. doses of doxapram, was made alkaline and the amines were separated by chloroform extraction. Thin layer chromatography of the concentrated chloroform solution revealed three spots which fluoresced after irradiation and which were positive to permanganate and ninhydrin sprays.

TABLE III

DISTRIBUTION OF DOXAPRAM-LIKE MATERIALS IN DOGS SACRIFICED FOUR HOURS FOLLOWING A SINGLE I.V. ADMINISTRATION OF 20 MG./KG. OF DOXAPRAM

	Dog									
	391-62	217-63	249-63	269-63	307-63	343-63	396-63	341-62[a]	Av.	341-62[b]
	Concentration, γ/g.									
Fat	32.4		13.5	24.9	7.8	17.0	22.9		19.8	25.6
Muscle	2.6	6.9	5.3	4.8	4.4	3.4	3.8	3.6	4.4	6.3
Spleen	4.2	13.4	14.3	16.7	8.5	5.0	3.2	9.2	9.3	13.0
Pancreas		12.9	13.7	8.7	10.2	11.9	2.6	16.1	10.9	24.2
Lung	3.8	12.7	10.0	10.5	6.2	5.0	4.8	20.2	9.2	29.0
Kidney	3.5	7.7	12.1	8.0	9.0	8.5	7.9	11.8	8.6	17.3
Small intestine		5.7	8.6	6.5	6.2	4.1	6.1	11.3	6.9	19.2
Large intestine		3.6	7.8	5.0	7.9	16.7	7.5		8.1	
Liver	6.9	15.4	14.9	24.6	18.6	14.0	6.8	44.6	18.2	62.4
Heart		7.1	6.9	5.9	4.1	1.7	4.9	9.4	5.9	11.7
Adrenal	11.5	6.5	13.2	17.4	16.8	6.0	2.2	5.5	9.8	7.0
Spinal cord	1.42	2.9	3.3	2.7	5.0	7.3	1.5	1.6	3.2	2.6
Medulla	1.0	4.6	2.8	4.7	2.0	3.7	3.9	2.6	3.2	3.0
Cerebellum		3.2	3.6		4.3	6.4	1.7	3.0	3.7	2.8
Cortex	2.4	3.4	2.3	4.2	3.0	5.5	1.9	3.2	3.2	2.9
Blood	1.4	3.0	1.6	3.9		1.0	0	2.5	2.0	3.7

[a] Direct oxidation and steam distillation method followed for these determinations. [b] Isotope method.

The R_f values of these spots did not correspond to the spot obtained from doxapram. For further identification, the spots were removed and oxidized by the analytical method. Each gave typical ultraviolet curves for benzophenone. Such analyses have repeatedly been carried out but unchanged doxapram has never been detected.

Since relatively large amounts of doxapram-like materials were found in the fat of dogs by the above analyses and no unchanged doxapram was found in the urine, it was interesting to determine whether it was present in fat. A sample of 53 g. of fat from dog 269-63 (containing 1.3 mg. of doxapram-like materials by analysis) was extracted with chloroform according to the analytical method. The extract was concentrated and chromatographed along with doxapram by the thin layer method on silica gel using ethanol and ethyl acetate–ammonium hydroxide (100:1 v./v.) as solvents. One spot containing doxapram-like materials was found in the fat extract, as revealed by the methods used for the above urine analysis. This spot did not correspond to doxapram and the structure of the compound has not been determined.

Treatment of a large sample of blood in the same manner as that described above also failed to show the presence of doxapram. Amine components which gave positive ninhydrin spots and which yielded benzophenone on oxidation were present.

A sample of blood was taken 30 min. after dosing from a dog that had received doxapram-C^{14}. Analysis of the sample showed 1.17 mg./100 ml. of doxapram or its metabolites was present. After centrifuging a portion, cells were separated from the plasma and each was analyzed. The plasma was found to contain 84% of the radioactive material. Repeated washing of the cells with saline failed to remove all of the radioactive material, indicating that some metabolic product must be firmly bound to the cells.

In order to attempt to determine what compound might be present in the blood, a 180-ml. sample, taken 30 min. after dosing with doxapram-C^{14}, was dialyzed against pH 7.4, 0.1 *M* phosphate buffer. The dialysate was evaporated to dryness under reduced pressure, the residue was taken up in a minimum volume of ethanol and chromatographed. A major radioactive spot was found at R_f 0.29. The R_f value for the ethanol amine derivative (see below) isolated from urine was 0.30, indicating that this compound may be present in appreciable amounts in the blood. No spot was found corresponding to doxapram.

In order to investigate further the nature of the metabolites present in urine, 48-hr. collections were made and partitioned into solubility classes. Basic and neutral materials were separated by making the urine alkaline and extracting (all extractions were carried out in continuous liquid–liquid extractors) with benzene until no more benzophenone-yielding materials appeared in the organic layer. The benzene solution was then extracted with 6 *N* HCl, by shaking, to separate neutral from basic components. The residual urine was acidified, while in an ice bath, and the urine again was extracted with benzene to separate acidic materials. Following this, the urine was hydrolyzed by refluxing for 3 hr., and again extracted while still acidic. The urine then was made alkaline and extracted again. Once again the urine was hydrolyzed and the alkaline and acidic extractions were repeated. The residual urine was neutralized and evaporated to dryness, and the residue was extracted with methanol. Each of the above extracts were analyzed by the chemical method. These results are presented in Table IV.

Doxapram-related compounds were found in all fractions except that following alkaline hydrolysis with

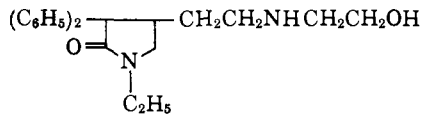
TABLE IV

PARTITIONING OF METABOLITES ACCORDING TO SOLUBILITY

	% of total		
Benzene soluble from	Dog urine	Dog bile	Human urine
Alkaline solution	49	12	46
Acid solution	9	7	10
Acid after acid hydrolysis	3	5	6
Alkali after acid hydrolysis	5	12	4
Acid after alkaline hydrolysis	4	2	2
Alkali after alkaline hydrolysis	0	0	0
Residual solution	19	23	15

alkaline extraction. A large number of metabolites, therefore, appeared to be present. The immediate suspicion was that extraction may have been incomplete. However, this did not appear to be likely since each extraction was carried until no more appeared in the extracting benzene and since the analytical method can determine as little as 5 γ. Lack of complete hydrolysis could have accounted for material being present after both alkaline and acid hydrolysis. These results indicated that at least one of each of the following classes of material was present: an amine, an acid, a "neutral" material, a conjugated amine, and a conjugated acid.

Thin layer chromatography in several solvents on silica gel, as stated above, indicated that three amines were present, none of which was doxapram. One of these amines, the one appearing in the largest amount, was isolated as follows: a chloroform solution of the fraction was acidified with concentrated hydrochloric acid and evaporated to dryness. The yellow oil was taken up in a small volume of ethanol and the amine precipitated by adding a large volume of isopropyl ether. The precipitate was filtered off and recrystallized six times from methanol–isobutyl methyl ketone. The resulting material proved to be identical with 1-ethyl-4-(2-hydroxyethylaminoethyl)-3,3-diphenyl-2-pyrrolidinone (II). Confirmation was ob-

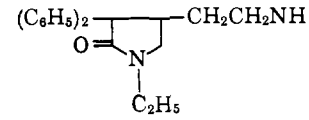


II

tained from mixture melting points, infrared spectra, t.l.c., and elemental analyses. Two previous reports have been found in the literature concerning the metabolism of morpholine[6a] and morpholine derivatives.[6b] Neither of these indicates opening of the morpholine ring, as was definitely shown by the above results.

Reaction of the spots obtained on t.l.c. with ninhydrin showed that one of the spots developed color much more rapidly and moved more slowly than did the spot corresponding to the above identified secondary amine. This led to the suspicion that the slower moving spot might be the corresponding primary amine. This amine [1-ethyl-4-(2-aminoethyl)-3,3-diphenyl-2-pyrrolidinone (III)] was synthesized and t.l.c. indicated that the two compounds were the same. Acetyl derivatives of each were prepared on thin layer plates by spotting each compound, the metabolic amine having been isolated by t.l.c., and then placing a drop of acetyl chloride on each. The excess acetyl chloride was allowed to evaporate overnight and the chromatograms developed with methanol–acetic acid (100:1). Both samples gave the same R_f value, which was different from that obtained from the unreacted amine. A larger amount of the mixed metabolic amines was separated by t.l.c. and the amine of interest was eluted from the silica gel with methanol. A KBr pellet was prepared and its infrared spectrum was compared with a corresponding pellet of the authentic primary amine. These also indicated that the compounds were the same.

(6) (a) R. K. Maller and C. Heidelberger, *Cancer Res.*, **17**, 296 (1957); (b) A. Englehardt, D. Jerchel, H. Weidman, and H. Wick, *Arch. Exptl. Pathol. Pharmakol.*, **235**, 10 (1958).



III

The relative quantities of the three amines detected were determined from thin layer chromatograms. The spots were identified by ninhydrin, removed from the plate, and analyzed. The results indicated that 50% of the amine fraction occurred as II and 25% as III, with the remainder being the unidentified amine.

The fraction obtained by extraction of the acidified urine was also investigated. Thin layer chromatography on silica gel using ethyl acetate containing 1% acetic acid for development revealed five spots that were positive to permanganate spray. One spot became fluorescent on irradiation with ultraviolet light. The spots were eluted and analyzed. Only one gave benzophenone on oxidation, indicating the presence of one acidic metabolite, with the diphenylmethyl moiety unchanged.

The presence of the 2-pyrrolidinone structure was indicated by an infrared curve obtained from material eluted from a thin layer chromatogram. This curve showed a band at 5.90 μ which is reported to be typical.[1]

Other fractions from the partitioning by solubility have not been investigated.

Bile Metabolites.—Bile samples (Table IV) were carried through the extraction procedure as described above for urine. The pattern of distribution appeared to be very similar to that for urine except that a smaller per cent of free amines and a larger per cent of conjugated amines were present. Aliquots of the free amine fraction were chromatographed and compared with the corresponding fraction obtained from urine. The chromatogram showed that the same three amines appeared in the bile that were present in the urine; a fourth amine was also present. However, none of these corresponded to unchanged doxapram. The ratios of the amounts of the three amines present, as indicated by analysis, were very similar to the ratio in urine.

Human Urine Metabolites.—Several samples of urine from patients who had received doxapram have been analyzed. The results are seen in Table V. Some of the administrations were by intravenous drip

TABLE V

URINARY EXCRETION OF DOXAPRAM-RELATED MATERIALS IN HUMANS

Dose, mg.	Period of collection, hr.	Doxapram-materials present	
		mg.	% of dose
5,320	0–36	455	8.6
2,840	0–40	244	8.6
2,000	0–24	204	10.2
49,000	0–72	1054	21.5

and some by injections. Interpretation is, therefore, difficult. The last patient reported was administered 2400, 1500, and 1000 mg. on three consecutive days with urine being collected at the same time. There was an increase in excretion each day, as would be expected.

One of the human urine samples was partitioned by the solubility method as described above. The pattern of distribution is shown in Table IV. Chromatog-

raphy of the amine fraction indicated that the same amines were present as in dog urine. Chromatography of the acid fraction indicated that the same acid was present.

Acknowledgment.—The authors gratefully acknowledge the assistance of other members of the research group at A. H. Robins Co., in particular, that of Dr. Bernard Franko and Miss Josephine Garber for the animal work and Dr. Herndon Jenkins for synthesis of compounds.

A Simple Test Tube Arrangement for Screening Fibrinolytic Activity of Synthetic Organic Compounds[1]

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Received August 17, 1964

It has previously been shown that a variety of synthetic organic compounds dissolve human plasma clots at a rather low concentration. A clear-cut relationship between chemical structure and activity was observed. Testing of activity, however, has required a complicated procedure. To avoid this difficulty, a new, simple, and quantitative screening test has been developed and is described in this paper. It consists essentially of the formation of clots from human plasma obtained from outdated blood bank blood. The clots are suspended in the solution of the compound to be studied and incubated for 24 hr. at 37°. With active compounds, the clots completely dissolved in this time. Based on the new screening test, some examples of the relationship of chemical structure and ability to dissolve clots are given for active benzoic acid derivatives, recently observed to induce fibrinolytic activity.

Development of cheap and reliable thrombolytic (clot dissolving) drugs which are easy to use is one of the great challenges in the field of therapeutics. At present, there are no drugs in wide-spread use which effectively cope with the number-one killer, intravascular clots. One major reason appears to be the lack of participation of the organic chemist in the development of fibrinolytic (thrombolytic) drugs which has been left entirely to the biochemist. The presently available enzymatic thrombolytic agents, though under certain circumstances quite useful, are generally disappointing from the clinical point of view: they are expensive, they require elaborate laboratory procedures for control, and they cannot be used on a preventive basis.

In this laboratory, it was discovered that a variety of organic compounds upon dissolution in human plasma in the test tube induced a marked fibrinolytic activity. This was not true of bovine, canine, and feline plasma. The ability of a compound to exhibit this particular property was structure dependent.[2-6] Minor changes of the chemical structure either enhanced or abolished the ability to produce fibrinolytic activity. The compounds were induced by a yet poorly understood pathway—activation of the fibrinolytic enzyme system in human plasma, demonstrated by fibrinolytic, caseolytic, and esterolytic activity.[2,6] Urethan was the first compound found to induce fibrinolytic activity in human plasma. Variations of the structure showed an increase of activity in this order: urethan < methylurethan < ethylurethan, and (for a related group of compounds) urea (no activity) < thiourea < ethylurea < allyl-2-thiourea.[4] As a working hypothesis it was assumed that hydrotropy could be the common denominator for the activity of these compounds. In order to test the hypothesis, other compounds with known hydrotropic activity but quite different structures were studied. Large asymmetric hydrotropic anions like 2,4-dimethylbenzenesulfonate and 2-naphthalenesulfonate brought about a 5-fold increase in activity as compared to urethan, whereas large asymmetric hydrotropic cations like tetra-*n*-propylammonium bromide were completely ineffective.[5] Compounds with a carboxyl group at the end of the hydrophilic side chain were slightly more active than the ones with sulfonic acid. Therefore, at present, our search for better compounds is concentrated on the former group. It has been possible by varying the structure further to increase the activity by a factor of approximately 40 and to define some additional structural requirements for an active compound (for examples see Discussion).[5,6] It is likely that intensive search for more active compounds will eventually lead to synthetic thrombolytic drugs. For these studies, a quantitative but rather elaborate testing procedure has been used previously.[7]

The investigations were hampered by the lack of quantitative testing methods suitable for screening and, most important, suitable for the researcher unfamiliar with the technique of blood coagulation and fibrinolysis. A new, simpler method, together with some instructive examples of test results, is briefly described in the Experimental section. It is hoped that the procedure will enable the organic chemist to participate in the quest for thrombolytic agents.

Experimental

Principle.—Human plasma clots are suspended at 37° in a solution of a compound to be tested. After 24-hr. incubation,

(1) Supported by a grant-in-aid of the American Heart Association, by Grant HE-05538 of the National Heart Institute, U. S. Public Health Service, and partly by a grant-in-aid of the Wyoming Heart Association.

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