Table V—Bismuth Subsalicylate Antidiarrheal Mixture Effect on Tetracycline Product Variability

Parameter	Range A	Range B	SD, % A	SD, % B
Peak serum level, μg/ml	0.69–2.29	1.79–2.52	24.4	9.34
Peak time, hr	2.50–4.0	2.50–5.0	18.9	37.6
AUC through 24 hr, μg/ml × hr	7.13–27.9	21.1–30.5	28.9	11.0
Urinary recovery through 72 hr, mg	42.2–173	108–216	32.8	18.8
Half-life, hr	7.09–10.8	7.58–11.8	11.4	15.1
Renal clearance, ml/min	53.3–151	25.8–126	29.7	27.3

suggested that the antidiarrheal influence on antibiotic absorption is dose dependent.

In the present study, 60 ml of a bismuth subsalicylate antidiarrheal product was administered concomitantly with a 250-mg tetracycline hydrochloride capsule. Since the "active" bismuth subsalicylate concentration was not given on the package, molar comparisons of "active" bismuth subsalicylate antidiarrheal to "active" kaolin–pectin products were impossible. Nonetheless, the 34% decrease in relative tetracycline bioavailability was consistent with the kaolin–pectin with tetracycline data and indicated that similar drug interactions are likely with any antidiarrheal whose mechanism of action is adsorptive.

REFERENCES

(1) J. G. Wagner, *Can. J. Pharm. Sci.*, **1**, 55 (1966).

(2) J. G. Wagner, *Drug Intell.*, **2**, 38 (1968).

(3) P. F. Binnion, in "Symposium on Digitalis," O. Storstein, Ed., Glydenal Norsk Forlag, Oslo, Norway, 1973, p. 216.

(4) D. D. Brown and R. P. Juhl, *N. Engl. J. Med.*, **295**, 1034 (1976).

(5) W. M. Gouda, *Abstr., Acad. Pharm. Sci.*, **6**, 117 (1976).

(6) K. A. DeSante, A. R. DiSanto, R. G. Stoll, R. D. Welch, T. J. Vecchio, and K. S. Albert, *ibid.*, **7**, 116 (1977).

(7) K. S. Albert, J. W. Ayres, D. J. Weidler, E. Sakmar, M. R. Hallmark, R. G. Stoll, K. A. DeSante, A. R. DiSanto, and J. G. Wagner, *ibid.*, **7**, 116 (1977).

(8) K. S. Albert, K. A. DeSante, R. D. Welch, and A. R. DiSanto, *ibid.*, **7**, 115 (1977).

(9) D. C. Grove and W. A. Randall, in "Medical Encyclopedia," H. Welch and F. Marti-Ibasey, Eds., Medical Encyclopedia Inc., New York, N.Y., 1955, p. 238.

(10) K. C. Kwan and A. E. Till, *J. Pharm. Sci.*, **62**, 1494 (1973).

ACKNOWLEDGMENTS

The authors thank Ms. Mariann Holthouse for secretarial assistance and W. L. Lummis for performing the microbiological assays.

Molecular Weight Determination of Commercial Heparin Sodium USP and Its Sterile Solutions

H. J. RODRIGUEZ * and A. J. VANDERWIELEN ˣ

Received June 19, 1978, from *Control Analytical Research and Development, The Upjohn Company, Kalamazoo, MI 49001.* Accepted for publication September 8, 1978. *Present address: Waters Associates, Milford, MA 01757.

Abstract □ A liquid chromatographic assay for the characterization of heparin sodium USP and heparin sterile solutions was developed. The method employs size exclusion chromatography and computer-based data collection and manipulation. An examination of commercially available heparin showed only minor differences between the heparins extracted from beef lung and porcine intestinal mucosa. The molecular weight averages of the material and its sterile solutions were 9000–12,000 daltons. A correlation was observed between average molecular weight and anticoagulant activity for the heparin sodium samples examined.

Keyphrases □ Heparin sodium—molecular weight determination of commercial products and sterile solutions using liquid chromatography □ Molecular weight determination—commercial heparin sodium USP and its sterile solutions, liquid chromatography □ Liquid chromatography—molecular weight determination of commercial heparin sodium USP and its sterile solutions □ Anticoagulants—heparin sodium, molecular weight determination, liquid chromatography

Several methods for the molecular weight fractionation of heparin sodium have been reported (1–3), gel filtration being most commonly used (4). In most cases, analysis requires several hours per sample and complicated detection procedures. Recently, a fast and reliable method was developed (5, 6) using high-performance liquid chromatography (HPLC) with refractive index detection for the determination of molecular weight averages of heparin sodium USP and injectable heparin sodium solutions (6).

The literature contains conflicting reports regarding the relationship of molecular weight and anticoagulant activity (7–9). There are also significant differences in the reported molecular weights of commercially available heparin (5, 6, 10). This paper presents the results of an examination of sterile injectable heparin sodium from commercial sources. The liquid chromatographic method recently developed (5) was modified and used to determine if any relationship exists between molecular weight and anticoagulant activity.

EXPERIMENTAL

A liquid chromatograph[1] was equipped with a refractive index detector and a syringe loop injector. A minicomputer[2] was used to monitor the refractive index detector signals and to run the molecular weight calculation programs. The detector–computer interface is shown in Fig. 1.

Chromatographic Conditions—Two sets of columns were used. The hand-packed set was used initially to compare molecular weight fractions for their relationship to anticoagulant activity. Commercially available columns were used for the comparative study on heparin sterile solutions.

A set of three columns was tap packed with various pore sizes of 5–10-μm glycophase-controlled porous glass. These columns were packed

[1] Waters Associates model ALC/GPC 244 equipped with a RI (R-404) and a U6K injector.

[2] PDP-11/40, Digital Equipment Co.; the interfacing was developed in-house.

0022-3549/79/0500-0588$01.00/0

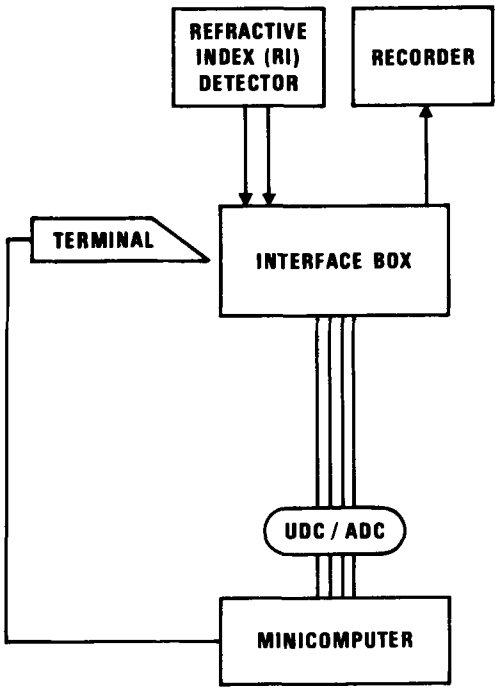

Figure 1—*Interface of refractive index detector to the minicomputer system. UDC = universal digital controller. ADC = analog to digital converter.*

by adding very small quantities (25–30 mg) of material and tapping the column 50–100 times. This procedure was repeated until the column appeared full. The column was then pressurized at 3500 psi, and any void observed was repacked. This procedure was repeated until no void was observed after pressurizing.

This column set consisted of three different lengths of 2.3-mm i.d. tubing packed as follows: 10 cm of 40-Å pore size, 25 cm of 100-Å pore size, and 60 cm of 250-Å pore size. The mobile phase used was 0.1 *M* sodium acetate (0.01% in sodium azide to inhibit bacterial growth) in double-distilled water at a flow of 0.1 ml/min (2500 psi).

All columns were calibrated using heparin sodium samples[3] of varied molecular weights. Retention times of these standards were plotted against molecular weight(s).

Efficient molecular sizing columns that can be used for molecular weight determinations are commercially available. These columns[4] consisted of 30-cm × 3.9-mm i.d. stainless steel tubing packed with microparticles (10 μm) of a fully porous silica–ether [$Si(RO)_xCH_3$].

After examination of various combinations of the available columns,

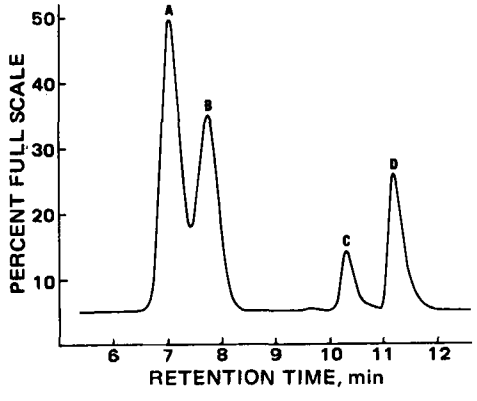

Figure 2—*Chromatogram of standard samples. Key: A, heparin of 5500 daltons (weight average); B, heparin of 12,100 daltons (weight average); C, injection artifact; and D, methanol. Full scale was 1.92 × 10^{-4} refractive index unit.*

[3] These standards (14K, 12K, 8K, and 5.5K) were obtained from J. A. Cifonelli.

[4] μBondagel, Waters Associates, Milford, MA 01757.

Table I—Calibration Results for Commercial Columns

Relative Retention Time[a] (Heparin–Methanol)	Molecular Weight, daltons × 1000
0.627	14.0
0.636	12.9
0.645	12.1
0.652	11.1
0.662	9.8
0.682	8.0
0.709	5.5

[a] Slope = −104.1, intercept = 79.8, correlation coefficient = 0.997, and estimated standard deviation of the line = 0.231.

a set consisting of one column of 125-Å pore size packing and two columns that were a blend of 125-, 300-, 500-, and 1000-Å pore size packing gave adequate resolution, reproducible measurements, and fast analyses (about 12 min/sample). A chromatogram showing the typical resolution obtained is shown in Fig. 2. This figure also shows the peaks due to methanol and an injection artifact resulting from changes in the refractive index due to inorganic salts (5, 6). Methanol (eluting at the total permeation volume) was used as an internal standard.

The columns were calibrated using seven samples of heparin sodium of known molecular weight. Three of these samples (14K, 8.0K, and 5.5K) were characterized previously using ultracentrifugation. The remaining samples were fractions collected on the original hand-packed columns, and their average molecular weights were determined on these columns (calibrated using four standards). The calibration was performed by a linear least-squares analysis on the relative retention time of heparin (relative to methanol) *versus* the weight average molecular weight. A well-characterized sample of sodium heparin was run with each set of analyses to monitor the performance of the columns and to correct the data for the comparative study (eliminate column and preparation variations).

Samples and Analysis—The samples consisted of commercially available material[5]. The powdered heparin sodium was dissolved (~12 mg/ml) in mobile phase containing 4% by volume methanol. Approximately 9 μl of this solution was injected. All sterile solutions were diluted accurately to a final concentration of 1000 USP units/ml by adding an appropriate amount of the mobile phase–methanol solution.

Molecular Weight Calculations—Molecular weight averages were calculated by literature methods (5, 11) using a FORTRAN program. The data were collected on-line by the system depicted in Fig. 1. The programs to collect the data and to perform the calculations were developed for this project to save time and calculation errors.

Anticoagulant Activity Determination—The samples were tested for potency (anticoagulant activity) using the USP assay (12).

RESULTS AND DISCUSSION

The results of a typical calibration are shown in Table I. Statistical treatment showed that molecular weight averages could be determined accurately to about ±500 daltons (*SD*) when the columns were calibrated properly. Figure 3 shows chromatograms of beef lung and intestinal porcine mucosal heparin. The chromatograms also indicate a difference that was noted previously (5): beef lung heparin sodium gave a fairly

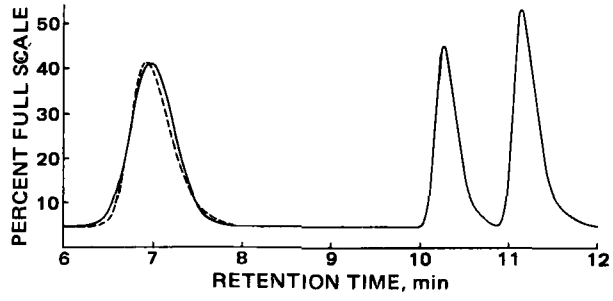

Figure 3—*Chromatograms of beef lung (—) and porcine intestinal mucosal (---) sodium heparin. Full scale was 0.48 × 10^{-4} refractive index unit.*

[5] The Upjohn Co., Riker Laboratories, Abbott Laboratories, Organon, Inolex, ASRY-Medical, and Pasadena Research Laboratories.

Table II—Comparison between Molecular Weight and Potency of Heparin Sodium Fractions

Fraction	Molecular Weight, weight average in daltons	Anticoagulant Activity, USP units/mg
1	13,800	167
2	12,900	157
3	12,100	155
4	11,100	145
5	9,800	124
6	9,100	100
7	8,000	76
8	7,500	50

Table III—Data on Heparin Sodium USP Grade Relation of Molecular Weight to Potency

Molecular Weight, daltons	Origin[a]	Anticoagulant Activity[b], USP units/mg
11,900	B	169
11,600	B	174
11,000	M	155–157
10,800	B, M	157–159
10,600	B, M	149–150
10,400	B	135–145
10,200	B	141–143
9,900	B	133–140
9,600	B	123–128

[a] B = beef lung, and M = porcine intestinal mucosa. [b] The ranges were obtained from two to four samples for each range.

symmetrical peak about the maxima whereas most porcine intestinal mucosal heparin gave a peak that was skewed toward low molecular weights.

Eight heparin fractions were collected using the hand-packed columns. When enough of each fraction had been collected, the heparin sodium was precipitated out of the mobile phase by adding methanol. In all cases, the heparin sodium precipitated at approximately a 1:2 ratio of methanol to mobile phase. After centrifuging and drying, the fractions were characterized accurately. The molecular weight was determined by the HPLC method, and the anticoagulant activities were determined by the USP method (Table II and Fig. 4).

These results show that there was a relationship between molecular weight and anticoagulant activity; however, this relationship was linear only over a narrow range of molecular weights (8000–12,000 daltons). Johnson and Mulloy (10), using intrinsic viscosity to measure molecular weight, observed this same relationship. In addition, they noted that the

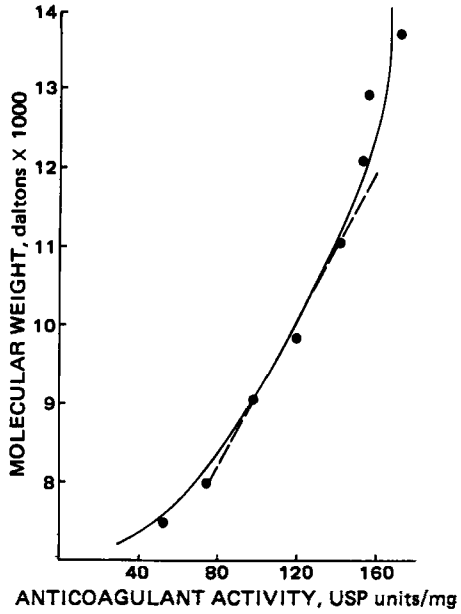

Figure 4—*Plot of anticoagulant activity of fractions* versus *molecular weight.*

Table IV—Molecular Weight of Commercial Heparin Sterile Solutions (1000 USP units/ml)

Sample[a]	Type	Molecular Weight, daltons: Weight Average	High	Low
A-1	Mucosal	10,400	15,400	2000
A-2	Mucosal	10,500	16,200	2500
A-3	Mucosal	10,100	16,800	2500
A-4	Mucosal	10,400	15,900	2400
X-1	Mucosal	10,900	16,100	2400
X-2	Mucosal	11,200	16,600	3500
Y	Mucosal	10,600	16,600	2400
D-1	Mucosal	10,900	17,300	4400
D-2	Beef lung	10,800	17,600	1700
W	Beef lung	10,600	16,900	1500
U-1	Beef lung	10,500	16,300	3700
U-2	Beef lung	10,200	16,200	2900

[a] Samples with the same letter designation are from the same commercial source.

Table V—Molecular Weight Comparison to Potency by Liquid Chromatography and Spectroscopy

Sample	Molecular Weight, daltons: HPLC	Spectroscopic[a]	Anticoagulant Activity, USP units/mg
CX-1	9,600	6,300	123
DF	10,000	7,600	136
CX-2	11,600	16,900	174
Average	10,400	10,300	

[a] Results obtained by Chromatrix, Sunnyvale, Calif., using a low-angle laser light-scattering technique.

anticoagulant activity decreased for molecular weights above 20,000 daltons.

The molecular weight and anticoagulant activity were determined on 32 different lots[6] of heparin sodium USP (24 of beef lung origin and eight of porcine intestinal mucosal origin). The results (Table III) show that the relationship observed for the heparin fractions also held true for the samples of commercial grade heparin. A linear regression analysis showed good correlation (0.93) and an estimated error of about 9 USP units (2 *SD*) for the anticoagulant activity. Although there was considerable overlap, these results indicate that one can expect differences in anticoagulants when the molecular weights differ by 1000 daltons or more.

In contrast to previously reported results (6), these results (Table III) show that beef lung heparin cannot be distinguished from porcine mucosal heparin on the basis of molecular weight analyses.

Twelve lots of sterile solution from the different manufacturers were analyzed by the HPLC method on the commercial columns. The results (Table IV) show a molecular weight range from 11,200 to 10,100 daltons. As noted previously (5), some lots of beef lung heparin had a distribution that was wider than porcine intestinal mucosal heparin (*e.g.*, compare Sample D-1 to D-2).

Sugisaka and Petracek recently (6) stated that porcine mucosal heparin has an average molecular weight somewhere between 21,000 and 24,000 daltons and that beef lung heparin has an average molecular weight between 16,000 and 19,000 daltons. This study shows that there is very little difference between commercial heparin extracted from beef lung and porcine intestinal mucosa and that the average molecular weight is 9000–12,000 daltons. The major difference between the results on the average molecular weight of sterile solutions is the calibration method. In this study, standardized heparin sodium was used to calibrate the liquid chromatographic column whereas Sugisaka and Petracek (6) used dextrans of various molecular weights.

The difference observed is not unexpected since molecular size is the property responsible for the separation observed by HPLC. Differences in molecular shape can appear as differences in molecular weight in size exclusion chromatography. The higher results obtained when the columns are calibrated with dextrans (6) are to be expected if the dextran molecules are smaller than molecules of heparin of equivalent weight. Accurate calibration, therefore, requires that the standards have the same molecular shape as the samples.

To check the accuracy of these results, the molecular weights of three samples were determined using a low-angle laser light-scattering tech-

[6] Manufactured by Upjohn, Abbott, and Inolex.

nique. This technique requires no reference to external standards since the molecular weight is obtained from a simple linear extrapolation to zero concentration of the light-scattering data obtained at a single angle from two or more solutions of different solute concentrations (13–16). The results (Table V) show that the HPLC molecular weight determinations were in fair agreement with those of the light-scattering technique. The average of the three samples agreed within 100 daltons, although there was considerable sample-to-sample variation. Both the HPLC and spectroscopic results showed a good correlation between molecular weight and anticoagulant activity.

REFERENCES

(1) G. Totolani and E. Romagnoli, *Anal. Biochem.*, **66**, 29 (1975).

(2) H. B. Nader, N. M. McDuffie, and C. P. Dietrich, *Biochem. Biophys. Res. Commun.*, **57**, 488 (1974).

(3) J. A. Cifonelli and J. King, *Biochim. Biophys. Acta*, **320**, 331 (1973).

(4) J. Ehrlich and S. S. Stavila, *J. Pharm. Sci.*, **62**, 617 (1973).

(5) H. J. Rodriguez, *Anal. Lett.*, **9**, 497 (1976).

(6) N. Sugisaka and F. J. Petracek, *Fed. Proc.*, **36**, 89 (1977).

(7) S. S. Stivala, L. Yuan, J. Echlich, and P. A. Liberti, *Biochem. Biophys.*, **122**, 32 (1967).

(8) G. H. Barlow, N. D. Sanderson, and P. G. McNeill, *Arch. Biochem. Biophys.*, **115**, 360 (1961).

(9) T. C. Laurent, *ibid.*, **92**, 224 (1961).

(10) E. A. Johnson and B. Mulloy, *Carbohydr. Res.*, **51**, 119 (1976).

(11) R. Guliana and L. Wild, *J. Polym. Sci., A2*, **5**, 1087 (1967).

(12) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, p. 229.

(13) W. Kaye and A. J. Havlik, *Appl. Opt.*, **12**, 541 (1973).

(14) H. J. Cantow, E. Siefert, and R. Kuhn, *Chem. Eng. Technol.*, **38**, 1032 (1966).

(15) A. C. Ouano and W. Kaye, *J. Polym. Sci.*, **12**, 1151 (1974).

(16) A. C. Ouano, *J. Chromatogr.*, **118**, 303 (1976).

ACKNOWLEDGMENTS

The authors thank Dr. Lloyd Fox and Mr. Norman Young for providing the interface for the refractive index detector and Mr. Bill Frailing for providing the FORTRAN programs for on-line data collection and manipulation. They also appreciate the assistance of P. A. Hartman, E. C. Berro, and M. C. Walker.

Heterocyclic Analogs of Amphetamine: Thioureas, Dithiocarbamates, and Negatively Substituted Amides

WILLIAM O. FOYE ˣ and SUCHINTA TOVIVICH

Received September 1, 1978, from the *Samuel M. Best Research Laboratory, Massachusetts College of Pharmacy, Boston, MA 02115.* Accepted for publication October 20, 1978.

Abstract □ A series of heterocyclic analogs of amphetamine was synthesized. The heterocycles employed included the 2-furyl, 2-thienyl, 3-methyl-2-thienyl, 3-pyridyl, and 6-methyl-2-pyridyl rings. The aliphatic amine group was converted to the *N*-methylthiourea, dithiocarbamate, methanesulfonyl, trifluoromethanesulfonyl, and trifluoroacetyl functions since similar conversions of the β-phenethylamine structure had shown blood pressure-lowering effects and some loss of behavioral effects. *p*-Chlorophenyl and 1-naphthyl analogs were also converted to these derivatives. Behavioral and other biological effects, including antiarthritic, passive cutaneous anaphylactic, and antimicrobial, were observed. The 3-methyl-2-thienyl analog of amphetamine significantly increased papillary muscle contractile force without producing arrhythmias.

Keyphrases □ Amphetamine analogs—thioureas, dithiocarbamates, negatively substituted amides, synthesis, behavioral effects, antiarthritis effects, cutaneous anaphylaxis, antimicrobial effects, blood pressure, rats □ Structure–activity relationships—amphetamine analogs, thioureas, dithiocarbamates, negatively substituted amides, synthesis, behavioral and biological effects, rats □ Motor activity—effects of amphetamine analogs, rats □ Antiarthritis agents—amphetamine analogs, rats □ Blood pressure—effects of amphetamine analogs, rats

Formation of negatively substituted amides on the aliphatic nitrogen of amphetamine and related structures has produced compounds with blood pressure-lowering effects (1). This result occurred also with negatively substituted amides of the β-phenethylamine structure as well as dithiocarbamate and thiourea derivatives (2, 3). Conversion of the aliphatic nitrogen to a neutral or acidic function thus led to a depressor response from a basic structure having motor stimulant activity. Previously, inclusion of the alkanesulfonamide group in the aromatic ring of phenethanolamines conferred either adrenergic stimulant or blocking activity (the latter generally appearing with an isopropyl or larger group on the aliphatic nitrogen) (4, 5).

DISCUSSION

A number of dithiocarbamates of the β-phenethylamine structure showed varying degrees of amphetamine-like behavioral effects (6). The presence of heterocyclic rings, in place of the phenyl, generally resulted in a loss of amphetamine behavioral effects. Diethyldithiocarbamate had previously been shown to deplete brain norepinephrine and to maintain the conditioned avoidance response of the rat (7). The dithiocarbamates of the heterocyclic ethylamines failed to alter this response (6). Antiobesity manifestations were observed in rats with some negatively substituted amides, dithiocarbamates, and thioureas of the β-phenethylamines and amphetamine derivatives. The possibility existed, therefore, of removing many or all of the behavioral attributes of the basic amphetamine structure while retaining depressor and antiobesity effects by appropriate substitutions on the nitrogen and inclusion of heterocyclic rings.

A series of heterocyclic analogs of amphetamine, including neutral or negatively substituted functions on the aliphatic nitrogen, was synthesized to determine if behavioral activity could be diminished and other useful effects retained. Corresponding derivatives of *p*-chloroamphetamine and the 1-naphthyl analog were prepared for similar observations. Negatively substituted amides prepared included the methanesulfonamide, trifluoromethanesulfonamide, and trifluoroacetyl as well as dithiocarbamates and methylthioureas. Both thioureas and dithiocarbamates have shown inhibitory activity against dopamine-β-hydroxylase (8, 9), so these derivatives were expected to produce depressor effects.

Previously, the furyl and thienyl analogs of amphetamine were obtained, and their motor effects were compared to those of amphetamine (10). Amphetamine and its thienyl analog were more active motor stimulants than the furyl analog, but both the heterocyclic analogs were less toxic than amphetamine.

0022-3549/79/0500-0591$01.00/0