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Synthesis of Substituted Anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes: Correlation between Anticonvulsant Activity and Monoamine Oxidase Inhibitory and Antihemolytic Properties

C. DWIVEDI*, RAYMOND D. HARBISON*, B. ALI‡, and SURENDRA S. PARMAR‡ˣ

Abstract □ Several substituted anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes were synthesized and characterized by their sharp melting points, elemental analyses, and IR spectra. Their ability to inhibit monoamine oxidase and to afford protection against hypoosmotic hemolysis in dog erythrocytes was found to bear no relationship with their anticonvulsant activity against pentylenetetrazol-induced seizures in mice.

Keyphrases □ Anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes, substituted—synthesis, characterization, monoamine oxidase, antihemolytic, and anticonvulsant activity □ Anticonvulsant activity—substituted benzylidenes, relationship to monoamine oxidase inhibitory and antihemolytic activity □ Antihemolytic activity—substituted benzylidenes against hypoosmotic hemolysis, relationship between monoamine oxidase inhibitory activity—substituted benzylidenes, relationship between antihemolytic and anticonvulsant activity

Hydrazine derivatives (1) and semicarbazides and thiosemicarbazides (2–4) have been shown to inhibit monoamine oxidase [EC 1.4.3.4 monoamine: O_2 oxidoreductase (deaminating)]. Monoamine oxidase inhibitors have also been shown to possess pronounced anticonvulsant properties (5). Evidence has been put forward to demonstrate that various drug responses are presumably mediated due to alterations in the physical properties of cell membrane and drug receptor interactions (6, 7). Studies with various central nervous system depressants, tranquilizers, and biogenic amines (8–11) revealed the ability of these compounds to stabilize the red blood cell membrane. Furthermore, substituted benzylidenes have been reported to possess psychotropic properties (12). These observations led to the synthesis of substituted anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes. The ability of these benzylidenes to inhibit monoamine oxidase and to exhibit membrane-stabilizing properties was investigated. The anticonvulsant activity of these compounds against pentylenetetrazol-induced seizures was also determined in an attempt to correlate their anticonvulsant activity with their monoamine oxidase inhibitory property and their ability to afford protection against hypoosmotic hemolysis using dog red blood cells.

The various substituted benzylidenes were synthesized by following the methods outlined in Scheme I.

EXPERIMENTAL[1]

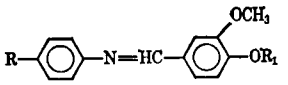
Chemistry—Substituted anilines (I) on condensation with vanillin (II) gave substituted anilino-(3-methoxy-4-hydroxy)benzylidenes (III and IV). Treatment of these compounds with ethyl chloroacetate resulted in the formation of substituted anilino-[3-methoxy-4-(ethoxycarbonylmethyleneoxy)]benzylidenes (V and VI) which, on treatment with hydrazine hydrate, gave substituted anilino-[3-methoxy-4-(hydrazinocarbonylmethyleneoxy)]benzylidenes (VII and VIII). These benzylidenes, on further treatment with aryl isothiocyanates, yielded substituted anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes (IX–XXIV).

Substituted Anilino-(3-methoxy-4-hydroxy)benzylidenes (III and IV)—A mixture of 0.1 mole of substituted aniline (I) and 0.1 mole of vanillin (II) in ethanol, together with a few drops of acetic acid, was refluxed for 4 hr. The solid mass, which separated on cooling, was filtered, dried, and recrystallized from ethanol (Table I).

Substituted Anilino-[3-methoxy-4-(ethoxycarbonylmethyleneoxy)]benzylidenes (V and VI)—To a solution of 0.08 mole of III and IV in dry acetone were added 0.08 mole of ethyl chloroacetate and 0.08 mole of anhydrous potassium carbonate, and the reaction mixture was refluxed on a steam bath for 15 hr. The reaction mixture was filtered and excess acetone was removed by distillation. The crude product, which separated on cooling, was filtered, dried, and recrystallized from ethanol (Table I).

[1] Analyses for carbon, hydrogen, and nitrogen were performed by the Central Drug Research Institute, Lucknow, India. Melting points were taken in open capillary tubes and are uncorrected.

Table I—Physical Constants of Substituted Anilino-3-methoxy-4-substituted Benzylidenes

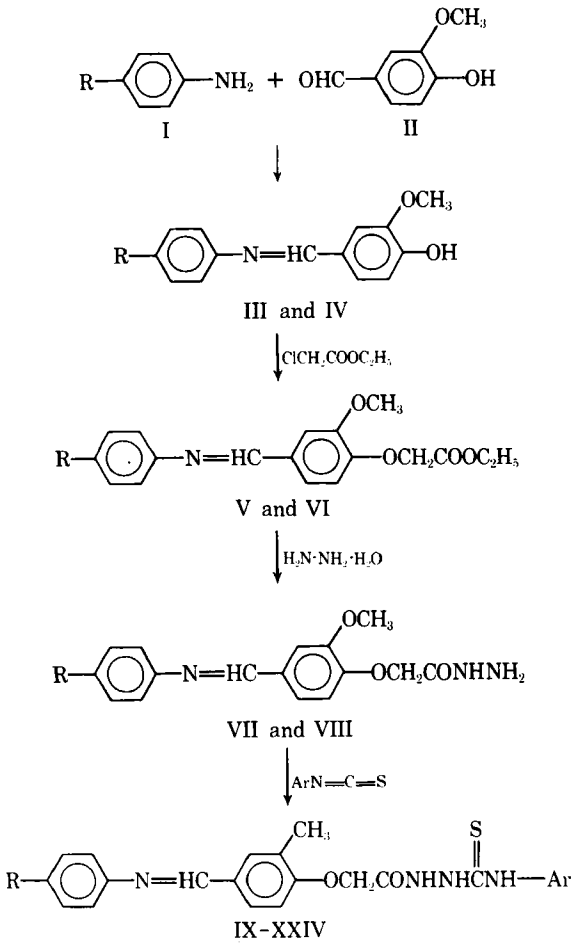


Compound	R	R_1	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Found
III	CH_3	H	120°	85	$C_{15}H_{15}NO_2$	C 74.68	74.92
						H 6.22	6.01
						N 5.80	5.65
IV	OCH_3	H	122°	84	$C_{15}H_{15}NO_3$	C 70.03	70.02
						H 5.83	5.76
						N 5.44	5.16
V	CH_3	$CH_2COOC_2H_5$	110°	80	$C_{19}H_{21}NO_4$	C 69.72	69.48
						H 6.42	6.51
						N 4.28	4.56
VI	OCH_3	$CH_2COOC_2H_5$	128°	80	$C_{19}H_{21}NO_5$	C 66.47	66.52
						H 6.12	5.06
						N 4.08	3.92
VII	CH_3	$CH_2CONHNH_2$	168°	65	$C_{17}H_{19}N_3O_3$	C 65.17	65.36
						H 6.07	5.92
						N 13.41	13.28
VIII	OCH_3	$CH_2CONHNH_2$	124°	62	$C_{17}H_{19}N_3O_4$	C 62.00	61.92
						H 5.77	6.02
						N 12.76	12.56

[a] All melting points were taken in open capillary tubes and are uncorrected.

Substituted Anilino-[3-methoxy-4-(hydrazinocarbonylmethyleneoxy)]benzylidenes (VII and VIII)—A mixture of 0.06 mole of V and VI and 99–100% hydrazine hydrate (0.09 mole) in absolute ethanol was refluxed on a steam bath for 12–15 hr. Excess ethanol was removed by distillation. The crude product, which separated on cooling, was filtered, dried, and recrystallized from benzene (Table I).

Substituted Anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes (IX–XXIV)—The substituted anilino-[3-methoxy-4-(hydrazinocarbonylmethyleneoxy)]-benzylidenes (VII and VIII) (0.004 mole) and suitable aryl isothiocyanates (0.004 mole) were mixed in dry benzene, and the mixture was refluxed on a steam bath for 3–5 hr. Excess benzene from the reaction mixture was removed by distillation under reduced pressure. The solid mass, which separated on cooling, was filtered, dried, and recrystallized from ethanol. Compounds IX–XXIV, thus synthesized, are recorded in Table II. The presence of the characteristic bands of the N—C(=S)—N (1490 cm^{-1}) and C=N (1640 cm^{-1}) groups in the IR spectra of these compounds provided further support in confirmation of their molecular structure.



Scheme I

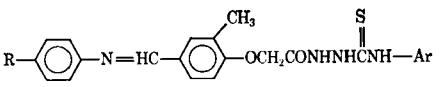
Determination of Monoamine Oxidase Activity—*Spectrophotofluorometric Method*—A spectrophotofluorometric method was used for the determination of monoamine oxidase activity of rat brain homogenate, using kynuramine as the substrate (13). The 4-hydroxyquinoline formed during oxidative deamination of kynuramine was measured fluorometrically in a spectrophotofluorometer[2] using activating light of 315 nm and measuring fluorescence at the maximum of 380 nm.

Male adult rats weighing approximately 150–200 g were killed by decapitation. Brains were quickly removed and homogenized[3] in ice-cold 0.25 *M* sucrose. The reaction mixture in a final concentration consisted of 0.5 ml phosphate buffer (0.2 *M*, pH 7.5), 1×10^{-4} *M* kynuramine, and 0.5 ml brain homogenate (equivalent to 10 mg of wet weight of the tissue). The monoamine oxidase activity of the brain homogenate was determined by incubation at 37° in air for 30 min. The various substituted benzylidenes were added to the brain homogenate to produce a final concentration of 5×10^{-5} *M* and incubated for 10 min before adding kynuramine. The mixture was then incubated for an additional 30 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (w/v), and the precipitated proteins were removed by centrifugation. Suitable 1-ml aliquots of the supernate were taken in 2 ml of 1 *N* NaOH solution and were assayed for 4-hydroxyquinoline. An increase in absorbance provided a direct measurement of the 4-hydroxyquinoline formation, which was taken as an index of the enzyme activity. The percent inhibition was calculated from the decrease observed in the absorbance and this provided an index of the inhibitory property of these substituted benzylidenes.

Warburg Manometric Method—The monoamine oxidase activity of rat brain homogenate was determined by the conventional Warburg manometric technique, using tyramine as the substrate (14). The decrease in oxygen uptake for 1 hr during oxidative deamination of tyramine in the presence of the substituted benzylidenes was used as an index of enzyme inhibition. The reaction mixture in a final concentration consisted of 50 m*M* phosphate

[2] Aminco-Bowman.
[3] Potter-Elvehjem homogenizer.

Table II—Physical Constants of 4-Substituted Anilino-[3-methoxy-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes



Compound	R	Ar	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Found
IX	CH_3	C_6H_5	120°	80	$C_{24}H_{24}N_4O_3S$	C 64.28	64.46
						H 5.35	5.29
						N 12.50	12.35
X	CH_3	2-CH_3—C_6H_4	175°	72	$C_{25}H_{26}N_4O_3S$	C 64.93	65.21
						H 5.62	5.54
						N 12.12	11.96
XI	CH_3	3-CH_3—C_6H_4	138°	74	$C_{25}H_{26}N_4O_3S$	C 64.93	64.74
						H 5.62	5.70
						N 12.12	12.02
XII	CH_3	4-CH_3—C_6H_4	160°	82	$C_{25}H_{26}N_4O_3S$	C 64.93	64.86
						H 5.62	5.58
						N 12.12	12.23
XIII	CH_3	4-OCH_3—C_6H_4	155°	81	$C_{25}H_{26}N_4O_4S$	C 62.67	62.82
						H 5.43	5.38
						N 11.71	11.63
XIV	CH_3	4-Cl—C_6H_4	156°	82	$C_{24}H_{23}ClN_4O_3S$	C 59.68	59.82
						H 4.76	4.62
						N 11.60	11.48
XV	CH_3	4-Br—C_6H_4	162°	84	$C_{24}H_{23}BrN_4O_3S$	C 54.64	54.38
						H 4.36	4.45
						N 10.62	10.56
XVI	CH_3	4-I—C_6H_4	166°	82	$C_{24}H_{23}IN_4O_3S$	C 50.17	49.98
						H 4.00	4.12
						N 9.77	9.58
XVII	OCH_3	C_6H_5	123°	79	$C_{24}H_{24}N_4O_4S$	C 62.06	61.82
						H 5.17	5.46
						N 12.06	11.98
XVIII	OCH_3	2-CH_3—C_6H_4	118°	73	$C_{25}H_{26}N_4O_4S$	C 62.76	63.05
						H 5.43	5.28
						N 11.71	11.46
XIX	OCH_3	3-CH_3—C_6H_4	135°	75	$C_{25}H_{26}N_4O_4S$	C 62.76	62.68
						H 5.43	5.52
						N 11.71	11.66
XX	OCH_3	4-CH_3—C_6H_4	130°	80	$C_{25}H_{26}N_4O_4S$	C 62.76	62.61
						H 5.43	5.38
						N 11.71	11.62
XXI	OCH_3	4-OCH_3—C_6H_4	156°	80	$C_{25}H_{26}N_4O_5S$	C 60.72	61.01
						H 5.26	5.12
						N 11.33	11.24
XXII	OCH_3	4-Cl—C_6H_4	158°	82	$C_{24}H_{23}ClN_4O_4S$	C 57.77	58.08
						H 4.61	4.48
						N 11.23	10.96
XXIII	OCH_3	4-Br—C_6H_4	166°	84	$C_{24}H_{23}BrN_4O_4S$	C 53.03	53.38
						H 4.23	4.16
						N 10.31	10.19
XXIV	OCH_3	4-I—C_6H_4	170°	83	$C_{24}H_{23}IN_4O_4S$	C 48.81	49.12
						H 3.89	3.66
						N 9.49	9.28

[a] All melting points were taken in open capillary tubes and are uncorrected.

buffer (pH 7.4), 5 m*M* tyramine, and the homogenate (equivalent to 250 mg of fresh tissue) in a total volume of 3.0 ml. The inhibitors, used at a final concentration of 1×10^{-3} *M*, were incubated with the enzyme preparation for 20 min before the substrate was added. The enzyme system was then incubated for an additional hour at 37°, using oxygen as the gaseous phase. Readings of oxygen uptake were made every 10 min.

Determination of Hypoosmotic Hemolysis—The procedure of Despopoulos (10), slightly modified, was used for the assay of hypoosmotic hemolysis; fresh blood of healthy dogs was used without oxygenation. An aliquot of 0.1 ml of dog blood was added to 3.0 ml of a buffer solution (0.425% Na_2HPO_4-NaH_2PO_4 buffer, 5 m*M*, pH 7.4; total osmolality 135 mosmoles/liter). The tubes, with and without the test compounds, were shaken gently and allowed to stand at room temperature for 10 min; then these tubes were centrifuged for 5 min at 1000×*g* to separate the cells. Under these conditions, hemolysis of the blood cells occurred but in no case exceeded 50%. Absorbance of the supernate was read at 540 nm in a colorimeter. Percent protection was calculated by comparing the absorbance values observed in the presence of the test compounds with that observed in the control tubes without the test compounds. The control value was represented as 100% hemolysis. Absorbance values for the blank containing 3 ml of 0.85% NaCl (normal saline) were subtracted from the values for both the control and experimental tubes. All test compounds were dissolved in the pH 7.4 phosphate buffer (osmolality 135 mosmoles) and were used at a final concentration of 1×10^{-4} *M*.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined (15) in mice of either sex weighing 25–30 g. The mice were divided in groups of 10, keeping the group weights as near the same as possible. Each benzylidene was suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compound was injected in a group of 10 animals at a dose of 100 mg/kg ip. Four hours after administration of the substituted benzylidenes, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol not only produced convulsions in almost all untreated mice but also exhibited 100% mortality during 24 hr. On the other hand, no mortality was observed during 24 hr in animals treated with 100 mg/kg alone of the test compounds. The mice were then observed 60 min for seizures. An episode of clonic spasm that persisted for a minimum of 5 sec was considered a threshold convulsion. Transient intermittent jerks or tremulousness was not counted. Animals devoid of threshold convulsions during 60 min were considered protected. The number of animals protected in each group was recorded and the anticonvulsant activity of these substituted

Table III—Monoamine Oxidase Inhibitory, Antihemolytic, and Anticonvulsant Properties of Substituted Anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes

Compound	Monoamine Oxidase Inhibition, %		Protection of Hypoosmotic Hemolysis[b], %	Anticonvulsant Activity[c], % Protection	Pentylenetetrazol Mortality, %
	Kynuramine[a]	Tyramine[a]			
IX	34.85 ± 0.56	30.61 ± 0.48	49.46 ± 0.75	50	10
X	46.91 ± 0.64	43.82 ± 0.56	43.17 ± 0.69	40	20
XI	38.87 ± 0.58	30.56 ± 0.47	47.96 ± 0.72	40	20
XII	32.17 ± 0.49	26.82 ± 0.43	52.66 ± 0.81	20	30
XIII	33.74 ± 0.52	21.90 ± 0.28	50.42 ± 0.80	60	10
XIV	68.25 ± 0.72	30.83 ± 0.36	41.04 ± 0.68	40	40
XV	64.34 ± 0.68	29.32 ± 0.18	48.18 ± 0.76	50	30
XVI	44.23 ± 0.59	27.35 ± 0.27	49.46 ± 0.88	60	20
XVII	33.51 ± 0.63	31.32 ± 0.42	31.37 ± 0.56	60	0
XVIII	36.19 ± 0.48	25.77 ± 0.38	63.83 ± 1.06	40	0
XIX	33.51 ± 0.42	37.20 ± 0.41	33.36 ± 0.58	40	10
XX	34.46 ± 0.46	31.38 ± 0.36	21.76 ± 0.54	70	0
XXI	32.17 ± 0.48	30.66 ± 0.44	34.75 ± 0.62	60	10
XXII	32.46 ± 0.50	40.68 ± 0.52	58.84 ± 0.78	80	0
XXIII	30.28 ± 0.40	35.29 ± 0.47	53.73 ± 0.69	60	0
XXIV	29.49 ± 0.32	25.68 ± 0.32	56.92 ± 0.76	60	0

[a] Compound numbers are those recorded in Table II. Each experiment was done in duplicate, and figures indicate mean values of three separate experiments with ± standard error of the mean. Vessel contents and assay procedures are as indicated in the text. The final concentrations of the inhibitors used were 5×10^{-5} and 1×10^{-3} *M* during oxidative deamination of kynuramine and tyramine, respectively. [b] Each experiment was done in duplicate and figures indicate mean values of three separate experiments with ± standard error of the mean. Assay procedure and contents of the reaction mixture are as indicated in the text. All compounds were used at a final concentration of 1×10^{-4} *M*. [c] Compounds were administered at a dose of 100 mg/kg ip. The screening procedure is as indicated in the text.

benzylidenes was represented as percent protection. The animals were then observed for 24 hr, and their mortality was recorded.

RESULTS AND DISCUSSION

In the present study, substituted anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes were tested for their monoamine oxidase inhibitory property, using kynuramine and tyramine as the substrates, and for their ability to inhibit the membrane-stabilizing properties of dog erythrocytes. The monoamine oxidase inhibitory property of these benzylidenes, at final concentrations of 1×10^{-3} *M* and 5×10^{-5} *M*, was determined during oxidative deamination of kynuramine and tyramine, respectively (Table III). Inhibition of the enzyme activity was significantly higher with kynuramine as compared to that with tyramine. Similar changes in the degree of monoamine oxidase have also been observed with other inhibitors during oxidative deamination of different substrates (4, 16, 17). The presence of different substituents on the phenyl group of the thiosemicarbazide moiety of both 4-methyl- and 4-methoxyanilinobenzylidenes influenced their ability to inhibit monoamine oxidase. The effect of a methyl group on the degree of monoamine oxidase inhibition was found to be in the order of 2-methyl > 3-methyl > 4-methyl substituent (X–XII and XVIII–XX). The 4-methoxy substituent produced a similar degree of enzyme inhibition, as was observed with compounds possessing an unsubstituted phenyl nucleus (IX, XIII, XVII, and XXI). Among compounds possessing a 4-halogen-substituted phenyl nucleus, 4-chlorophenyl substituted compounds (XIV and XXII) produced a greater degree of monoamine oxidase inhibition than the corresponding 4-bromophenyl- or 4-iodophenyl-substituted compounds (XV, XVI, XXIII, and XXIV).

All substituted benzylidenes were found to protect red blood cells against hypoosmotic hemolysis (Table III). Introduction of various substituents at position 4 of the thiosemicarbazide moiety of 4-methylanilinobenzylidenes produced no significant effect on their ability to protect against hemolysis (IX–XVI). Maximum protection was observed with Compound XII. On the other hand, attachment of the various substituents at position 4 of the thiosemicarbazide moiety of 4-methoxyanilinobenzylidenes was found to play a definite role in their antihemolytic activity. The presence of the 2-methylphenyl substituent at position 4 was found to be most effective in exhibiting the antihemolytic property (XVIII). Variations in the position of the methyl group of the phenyl substituent attached to position 4 of the thiosemicarbazide moiety decreased the antihemolytic activity (XIX and XX). A similar decrease was also observed on the introduction of the 4-methoxyphenyl group at position 4 of the thiosemicarbazide moiety (XXI). On the other hand, the presence of a 4-halosubstituted group at position 4 of the thiosemicarbazide moiety produced increased protection against hypoosmotic hemolysis (XXII–XXIV).

The anticonvulsant activity observed with these benzylidenes at a dose of 100 mg/kg against pentylenetetrazol-induced seizures ranged from 20 to 80% (Table III). Maximum protection was observed with Compound XXII. As is evident from Table III, all 4-methoxyanilinobenzylidenes (XVII–XXIV) not only elicited a relatively higher degree of protection but also protected the experimental animals against pentylenetetrazol-induced mortality during 24 hr. These observations do not prove that the membrane stabilization and monoamine oxidase inhibitory properties of these substituted benzylidenes are the basis for their anticonvulsant activity. Further studies dealing with pharmacological and toxicological properties and their effects upon the activity of other purified enzyme preparations may possibly reflect a biochemical basis for the anticonvulsant activity of these substituted benzylidenes.

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PHARMACEUTICAL ANALYSIS

Determination of Silicon in Streptomycin by Atomic Absorption

R. J. HURTUBISE

Abstract □ An atomic absorption method was developed for determining silicon in streptomycin without prior separation. The method has a reproducibility of ±2.2 ppm and a detection limit of 5 ppm silicon. Ash-fused and untreated samples were compared, and a deuterium arc background corrector indicated a moderately high bias in the results.

Keyphrases □ Silicon—atomic absorption determination in streptomycin □ Streptomycin—atomic absorption determination of silicon content □ Atomic absorption spectroscopy—determination of silicon in streptomycin

Because the manufacturing of pharmaceuticals involves many complex chemical and physical processes, contaminants can be introduced in many places before the final product is obtained. Thus, there is a need for precise, accurate, and sensitive analytical methods to determine contaminants. Silicon can be introduced by raw materials, water, and other sources into many in-process manufacturing samples. In the pharmaceutical industry, the control of the amount of silicon is important because it can directly or indirectly cause turbidity in solutions of product samples.

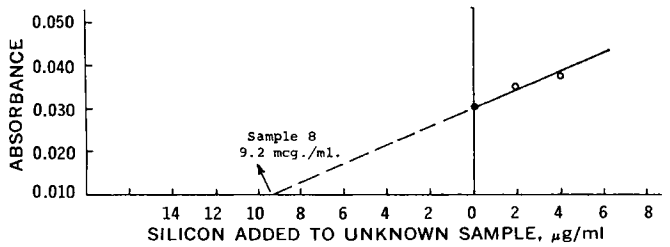

Figure 1—*Typical standard addition curve for streptomycin.*

Few atomic absorption methods have been published for the determination of silicon in pharmaceuticals (1, 2). This paper describes a rapid, sensitive, and accurate atomic absorption method for the determination of silicon in the antibiotic, streptomycin. Ashing and alkaline fusion steps are not needed, and a deuterium arc background corrector may be employed, depending on the accuracy desired.

EXPERIMENTAL

Apparatus—An atomic absorption spectrophotometer[1] equipped with a nitrous oxide burner and a deuterium arc background corrector[2] were used according to the manufacturer's instructions. A single-element silicon hollow-cathode lamp[3] was used for all silicon determinations. The following instrument settings were used for all measurements: resonance line, 251.6 nm; slit 4; lamp current, 40 mamp; nitrous oxide flow, 13 liters/min; and acetylene flow, 12 liters/min. Experimental results indicated that Pyrex volumetric glassware was suitable for this work.

Reagents—Fisher certified sodium silicate atomic absorption standard was used to prepare all silicon standards.

Procedures—*Determination of Silicon*—Between 24.0 and 26.0 g of streptomycin sulfate was weighed accurately into a 50-ml volumetric flask and diluted to volume with distilled water. Ten-milliliter aliquots of this solution were transferred quantitatively to three 25-ml volumetric flasks. Aliquots (1 and 2 ml) of 50 μg/ml standard sodium silicate solution were added to two of the volumetric flasks, respectively, and the contents of all three flasks were diluted to volume with distilled water. The atomic absorption unit was adjusted so there was a fuel-rich flame, the absorbance was set at zero with distilled water, and the absorbance of the three solutions was determined. A standard addition curve was prepared by plotting absorbance against micrograms of silicon per milliliter added to streptomycin solutions. The line inter-

[1] Perkin-Elmer model 403.
[2] Perkin-Elmer.
[3] P-E Intensitron lamp.