Inhibition of Drug Metabolism

II. Metabolism of 2-Diethylaminoethyl 2,2-Diphenylvalerate HCl (SKF 525-A)

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SUMMARY

The metabolism of SKF 525-A by washed hepatic microsomes from the rat was studied. The secondary amine derivative, 2-ethylaminoethyl 2,2-diphenylvalerate HCl (SKF 8742-A) was identified as a metabolite. A second metabolite, designated metabolite I, was also observed. The acidic hydrolysis product of SKF 525-A, 2,2-diphenylvalerate (SKF 2314), was identified and the rate of hydrolysis of SKF 525-A by washed microsomes was measured. Metabolite I was not found in microsomal preparations that had been incubated with SKF 8742-A. No SKF 525-A or SKF 8742-A was recovered from the urine, bile, or feces of rats that had received these compounds, nor were metabolites found in these materials. The primary amine analog of SKF 525-A, 2-aminoethyl 2,2-diphenylvalerate hydrobromide (AEDV) was synthesized. AEDV was shown to be a competitive inhibitor of the N-demethylation of ethylmorphine with an inhibition constant quite similar to those of SKF 525-A and SKF 8742-A.

INTRODUCTION

In the preceding communication (1) the N-dealkylation of 2-diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A) by hepatic microsomes was studied, as was the N-dealkylation of the product of this reaction, the secondary amine, 2-ethylaminoethyl 2,2-diphenylvalerate HCl (SKF 8742-A). Studies of the effects of both compounds on the microsomal N-demethylation of ethylmorphine showed the secondary amine to be about as effective an inhibitor as SKF 525-A. In the current study, the primary amine analog of SKF 525-A, aminoethyl 2,2-diphenylvalerate HBr (AEDV), was synthesized and tested as an inhibitor of the microsomal N-demethylation of ethylmorphine. An attempt was made to identify the metabolic products of SKF 525-A and SKF 8742-A in extracts of microsomal incubation mixtures as well as in the urine, bile, and feces of rats that had received these compounds. The role that the acid hydrolysis product of SKF 525-A might play in the inhibition of drug metabolism was also investigated.

MATERIALS AND METHODS

Synthesis of 2-aminoethyl 2,2-diphenylvalerate hydrobromide. The synthesis of 2-aminoethyl 2,2-diphenylvalerate HBr (AEDV) by the condensation of 2,2-diphenylvaleryl chloride and ethanolamine was not attempted because the major product probably would have been the amide rather than the desired ester. Furthermore, if formed, the ester might be susceptible to a base-catalyzed rearrangement to the amide. Such difficulties were circumvented by first blocking the amino group of ethanolamine with a carbobenzoxy group. The ester was then formed by the conden-

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sation of the alcohol with acyl halide followed by the removal of the carbobenzoxy group under acid conditions so as to prevent the base-catalyzed rearrangement to the amide and at the same time to form the desired salt of the amine.

The carbobenzoxyethanolamine precursor was synthesized by the following procedure. Ethanolamine, 6.1 g (0.1 mole), was added to a suspension of 21.0 g (0.25 mole) of sodium bicarbonate in 125 ml of water and the suspension was stirred vigorously with a magnetic stirrer. Carbobenzoxy chloride, 17.2 g (0.11 mole), was added to the suspension in 5 portions over a 30-min period, and the mixture was then stirred for 1 hr during which time an oil formed. The mixture was acidified with 5 N hydrochloric acid solution until no more gas evolved and then placed in the cold overnight. The resulting crystals were collected, the filtrate was concentrated to half its volume *in vacuo;* on cooling, a second crop of crystals was obtained. Recrystallization from benzene resulted in a yield of 90% carbobenzoxyethanolamine; m.p. 61–62° (uncorrected).

The 2,2-diphenylvaleryl chloride precursor was prepared by refluxing 6.35 g (0.025 mole) of 2,2-diphenylvaleric acid (SKF 2314)[2] with 10 ml (0.14 mole) of thionyl chloride under a reflux condenser fitted with a calcium chloride drying tube. After 8 hr the excess thionyl chloride was removed *in vacuo*. Infrared examination of the resulting oil indicated a yield of approximately 90% of 2,2-diphenylvaleryl chloride.

Carbobenzoxy-2-aminoethyl 2,2-diphenylvalerate was prepared by the condensation of 2,2-diphenylvaleryl chloride and carbobenzoxyethanolamine. Carbobenzoxyethanolamine, 3.7 g (0.019 mole dissolved in 4 ml of dry pyridine, and 2,2-diphenylvaleryl chloride, about 6.1 g (0.021 mole) dissolved in 2 ml of dry pyridine, were mixed and allowed to stand overnight in a stoppered vessel. The following morning the mixture was refluxed on a steam bath for 2 hr using a condenser fitted with a calcium chloride drying tube. The mixture was cooled and poured into 10 ml of 10% hydrochloric acid solution containing ice chips. This solution was extracted twice with 50 ml volumes of ether, the combined ether layers were washed twice with 10 ml volumes of 10% hydrochloric acid solution followed by 2 washes with 10 ml volumes of 5% sodium bicarbonate solution and a wash with 10 ml of water. The ether layer was dried over anhydrous sodium sulfate, filtered and evaporated to dryness *in vacuo*. The resulting oil, which was dried by adding small quantities of benzene and evaporating to dryness with the aid of a vacuum, was not purified further.

The carbobenzoxy blocking group was removed by nonhydrolytic cleavage with acid. Carbobenzoxy-2-aminoethyl 2,2-diphenylvalerate, obtained as an oil from the previous step, was added to 20 g of glacial acetic acid saturated with dry hydrogen bromide in a vessel fitted with a calcium chloride drying tube. After the evolution of carbon dioxide had ceased (about 3 hr) 100 ml of dry ether was added to precipitate the hydrobromide salt of the product. The mixture was stored overnight in the cold, filtered and dried *in vacuo* at room temperature. The pink crystals obtained were dissolved in hot toluene:chloroform (9:1), treated with decolorizing carbon and filtered while hot. The clear filtrate was cooled and the resulting white crystals were collected. Recrystallization from boiling toluene yielded 3.9 g (41% yield) of 2-aminoethyl 2,2-diphenylvalerate hydrobromide; m.p. 146°C (uncorrected). Calculated for $C_{19}H_{24}BrNO_2$: C, 60.33; H, 6.35; N, 3.70. Found: C, 59.71; H. 6.42; N, 3.67.

The base-catalyzed rearrangement of 2-aminoethyl 2,2-diphenylvalerate to *N*-(2-hydroxyethyl)-2,2-diphenylvaleramide (SKF 22983)[2] was used to confirm the synthesis. Seventy-five milligrams (0.20 mmole) of 2-aminoethyl 2,2-diphenylvalerate hydrobromide was mixed with 1 ml of 5 N sodium hydroxide solution, heated for 2 hr on a steam bath and allowed to stand overnight at room temperature. The mixture was extracted 3 times with 25 ml volumes of chloroform and the chloroform

[2] Supplied by Smith Kline & French Laboratories, Philadelphia, Pennsylvania.

extracts were combined and washed with 5 ml of 10% hydrochloric acid solution. The chloroform layer was filtered through anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The residue was dried by repeatedly evaporating small quantities of added benzene. Recrystallization from heptane gave white crystals, m.p. 86–87°. Mixed m.p. 86–87° (all m.p. uncorrected). When examined by gas–liquid chromatography, the retention times of both SKF 22983 and the rearranged product were identical.

SKF 525-A[2] and SKF 8742-A[2] were used as obtained from the suppliers without further purification.

Tissue preparation, incubation mixture and determination of enzyme activity. The preparation of washed hepatic microsomes, the contents of the incubation mixture and the methods for the determination of the rate of N-demethylation of ethylmorphine and the inhibition kinetics of that reaction have been described previously (1). When metabolites were sought, SKF 525-A and SKF 8742-A (4×10^{-4} M) were incubated with microsomal preparations for 15 min and then extracted immediately.

In vivo studies. Male Holtzman rats weighing 150–200 g were injected intraperitoneally with 50 mg/kg of either SKF 525-A or SKF 8742-A, and their urines and feces were collected for 24 hr. Bile was collected by cannulating the bile duct.

Extraction procedures. Metabolites formed by the incubation of SKF 525-A and SKF 8742-A with hepatic microsomes were extracted from the incubation mixtures with 10 volumes of chloroform. The chloroform layer was dried by filtration through anhydrous sodium sulfate, evaporated to dryness under a stream of nitrogen in a boiling water bath, and taken up in a small volume of methanol.

Urine was divided into two portions. Half was autoclaved at 15 pounds pressure for 30 min with one-tenth its volume of concentrated hydrochloric acid. This procedure did not alter the recovery of SKF 525-A added to the urine from control rats. An excess of solid sodium bicarbonate was added to both the autoclaved and unautoclaved urine samples, which were then extracted with five volumes of chloroform. The chloroform was removed by evaporation in a boiling water bath. The residue was dissolved in a small volume of methanol and subjected to thin-layer chromatography. Feces were dispersed in water with the aid of a Waring blender and treated in a manner similar to that described for urine. Similar extracts of hydrolyzed and unhydrolyzed bile were also prepared.

Hydrolysis of SKF 525-A by microsomal enzymes. The rate of hydrolysis of SKF 525-A was determined by measuring the appearance of the acidic product, 2,2-diphenylvaleric acid (SKF 2314). SKF 525-A (2×10^{-3} M) was incubated for 15 min with washed hepatic microsomes. The incubation mixture was extracted with 5 volumes of chloroform. The chloroform was removed by evaporation in a boiling water bath, and the residue was treated with 1 ml of a 1% alcohol-free ethereal diazomethane solution to form methyl 2,2-diphenylvalerate. The ethereal solution was then evaporated to dryness with nitrogen and the residue was taken up in a small volume of ethyl acetate and subjected to gas–liquid chromatography. Recovery of SKF 2314 added to incubation mixtures averaged 80% as measured by gas–liquid chromatography. The quantitative determination of methyl 2,2-diphenylvalerate was accomplished by measuring peak areas and comparing these with peak areas obtained from known quantities of the ester.

Thin-layer chromatography. Thin-layer chromatoplates were prepared by the method of Lees and DeMuria (2) using silica gel G (Merck) as the absorbent. The plates were air-dried, placed in an oven for 30 min at 80° and stored in a desiccator. Two solvent systems were employed (3); the first (S-1) consisted of ethanol, acetic acid, and water (60:30:10, v/v/v) and the second (S-2) of ethanol, pyridine, dioxane, and water (50:20:25:5, v/v/v/v). After development, the plates were air-dried and then dried in an oven at 80–100°. Spots were located by spraying with a potassium iodoplatinate reagent for amines

(4), Folin-Ciocalteu reagent (5) for phenols, or a sodium nitroprusside-acetaldehyde reagent for secondary amines (6).

Gas–liquid chromatography. A Barber-Colman Model 10 gas chromatograph equipped with a ^{90}Sr argon ionization detector was used. The column was a 6 ft by 6 mm, i.d., U-shaped borosilicate glass tube with a column packing consisting of the dimethyl polysiloxane polymer, SE-30, as the liquid phase and a silanized diatomaceous earth, Gas-Chrom S, as the solid support. The packing material was prepared as follows: 50 g of resieved 80–100 mesh Gas-Chrom S was digested with concentrated hydrochloric acid overnight at room temperature. The Gas-Chrom S was washed with distilled water by decantation and finally by filtration until the filtrate was free of chloride ions. After it had dried overnight at 105°, the material was suspended in 250 ml of a 10% solution of dimethyldichlorosilane in toluene for 30 min with periodic degassing with the aid of a vacuum. The mixture was then filtered with suction on a Büchner funnel and washed with 500 ml of toluene followed by 1 liter of anhydrous methanol. The packing material was dried overnight at 105° and then suspended in 500 ml of a 2% solution of SE-30 in toluene for 30 min with degassing. The mixture was filtered with suction and dried in an oven at 105°. The column was packed and then conditioned overnight at 275° with the carrier gas flowing. The column temperature was 175° when methyl 2,2-diphenylvalerate was studied and 190° when the other metabolites were under investigation. The flash heater and detector were maintained at 255°. The detector was operated at 1000 volts and the relative gain was 10^{-7} ampere. The inlet pressure was 20 psig and the outlet pressure was atmospheric. Metabolites were collected from the effluent of the argon ionization detector by condensing the argon with liquid nitrogen (7). The peak shift technique whereby derivatives are formed on the column (8) was used in the identification of metabolites. Volumes of 5 μl of acetic or propionic anhydrides, 1 μl of trifluoroacetic anhydride, and 5 μl of acetone were employed for the formation of derivatives on the column.

RESULTS

Metabolism of SKF 525-A

Gas–liquid chromatography of extracts of microsomal preparations that had been incubated with SKF 525-A revealed two peaks in addition to that produced by SKF 525-A (Fig. 1). These peaks were not derived from incubation mixtures to which no SKF 525-A had been added. The two peaks, with retention times of 0.48 and 0.69 relative to that of SKF 525-A, were

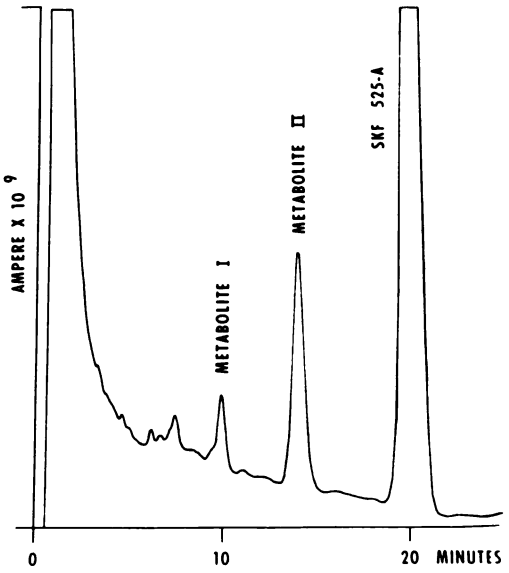

FIG. 1. *Gas-liquid chromatogram of SKF 525-A and its metabolites*

designated metabolite I and metabolite II, respectively. Column effluents represented by metabolite I and II peaks were trapped, and the residues thus obtained were subjected to further testing. Attempts were made to identify these metabolites by forming derivatives on the column (8). Anhydrides injected directly on the column will form esters with phenolic and alcoholic hydroxyl groups and amides with primary and secondary amines. Acetone reacts with primary amines to form Schiff base derivatives. The peak shifts effected

TABLE 1
Gas chromatography of SKF 525-A, its metabolites and their derivatives[a]

Compound	Peak-shifting reagent				
	None	Acetic anhydride	Propionic anhydride	Trifluoroacetic anhydride	Acetone
SKF 525-A	1.00	1.00	1.00	1.00	1.00
SKF 8742-A	0.70	2.79	3.30	1.32	0.71
AEDV	0.52	1.85	2.34	0.76	1.02
Metabolite I	0.48	0.48	0.48	0.38	0.48
Metabolite II	0.69	2.76	3.22	1.26	0.71

[a] Values are given as retention times relative to that of SKF 525-A (20.5 min).

by the injection of acetic anhydride, propionic anhydride, trifluoroacetic anhydride, and acetone are shown in Table 1. In accordance with expectations, no shift of the SKF 525-A peak was effected by any of the four reagents. SKF 8742-A and metabolite II formed derivatives on the column with acetic, propionic, and trifluoroacetic anhydrides which showed corresponding retention times. It is concluded from these results that SKF 8742-A and metabolite II are identical. The studies employing thin-layer chromatography reinforced this conclusion. The R_F values in the two solvent systems and the responses to the spray reagents were the same for SKF 8742-A and metabolite II (Table 2).

Metabolite I and AEDV had similar retention times, but they behaved differently when examined by the peak shift technique (Table 1). AEDV formed a derivative with acetone, metabolite I did not; this not only shows that the compounds are different, but that metabolite I is probably not a primary amine. That the formation of a derivative of metabolite I with trifluoroacetic acid has a shorter retention time than that of its parent compound suggests the presence of a hydroxyl group. Trifluoroacetic esters of phenols are known to have shorter retention times than their parent compounds (8, 9). If an amide had been formed its retention time would be expected to be longer than that of metabolite I. That an ester should form with trifluoroacetic anhydride, but not with acetic or propionic anhydride, is not necessarily inconsistent with the view that a hydroxyl group is involved; esters are not always formed on the column and an ester would be expected to form more readily with trifluoroacetic anhydride than with either acetic or propionic anhydrides. Thin-layer chromatograms of metabolite I (Table 2) showed no

TABLE 2
Thin-layer chromatography of SKF 525-A and its metabolites

Compound	R_F values		Reactions with spray reagents		
	S-1[a]	S-2[b]	Iodoplatinate	Folin-Ciocalteu	Nitroprusside acetaldehyde
SKF 525-A	0.74	0.75	+	−	−
SKF 8742-A	0.85	0.66	+	−	+
AEDV	0.87	0.75	+	−	−
Metabolite I	0.69	0.85	−	+	NT[c]
Metabolite II	0.84	0.65	+	−	+

[a] Solvent 1—ethanol:acetic acid:H_2O (60:30:10).
[b] Solvent 2—ethanol:pyridine:dioxane:H_2O (50:20:25:5).
[c] Not tested.

spots when sprayed with the iodoplatinate reagent, but a spot was obtained with the Folin-Ciocalteu phenol reagent, suggesting the presence of phenolic hydroxyl group. The failure to form a color with the iodoplatinate reagent does not exclude the possibility that metabolite I is an amine since some amines react poorly with this reagent and the quantities of the metabolite used in these studies may not have been sufficient to produce the color reaction. That metabolite I is not AEDV is again apparent.

The rate of hydrolysis of SKF 525-A by washed hepatic microsomes as determined by gas–liquid chromatography of the methyl ester of the acidic product was found to be 0.27 ± 0.02 μmoles per gram of liver per hour.

Thin-layer chromatographic examination of the extracts from both acid-hydrolyzed and unhydrolyzed urine, feces, and bile obtained from rats given SKF 525-A did not show the presence of SKF 525-A, SKF 8742-A or metabolite I. Gas–liquid chromatographic analysis of extracts of unhydrolyzed urine from rats given SKF 525-A failed to reveal the presence of the acidic product of hydrolysis (SKF 2314) although SKF 2314 was readily detected in the urine of rats given this compound (50 mg/kg).

Metabolism of SKF 8242-A

Gas–liquid chromatography of extracts of microsomal preparations that had been incubated with SKF 8742-A revealed the presence of SKF 8742-A only. The *in vivo* studies employing SKF 8742-A gave results similar to those obtained with SKF 525-A; no SKF 8742-A was detected in the urine, feces, or bile.

Inhibition of the N-demethylation of Ethylmorphine by AEDV

AEDV in concentrations of 1 and 5 $\times 10^{-5}$ M was tested as an inhibitor of the N-demethylation of ethylmorphine as described previously (1). AEDV was found to inhibit the reaction competitively with an inhibition constant (K_i) of 1.5×10^{-6} M.

DISCUSSION

The propensity for SKF 525-A to bind strongly to microsomal protein (10) creates some problems when the metabolism of this compound is studied. Preliminary experiments performed in this laboratory showed that SKF 8742-A and AEDV are also bound firmly. This may account for the failure to detect AEDV in the residues obtained from the extraction of microsomal preparations that had been incubated with either SKF 525-A or SKF 8742-A. It is conceivable that other metabolites may have been formed but remained so strongly attached to the protein that they were not extractable.

On the basis of a comparison of the K_i values obtained at 7.5- and 15-min incubation periods, where a significant decrease in the K_i value was noted at the longer time interval, it was suggested that a metabolite of SKF 525-A might be formed which is a more potent inhibitor of the N-demethylation of ethylmorphine than SKF 525-A itself (1). Because of the similarity of the K_i values of SKF 525-A and SKF 8742-A, 6.0×10^{-6} M and 3.6×10^{-6} M, respectively (1), SKF 8742-A is not thought to be the metabolite in question. The metabolite would not appear to be AEDV. If it were, a similar difference in K_i values should have been observed when SKF 8742-A was incubated for 7.5 and 15 min, and this was not the case. Because metabolite I is formed from SKF 525-A and not from SKF 8742-A, and because the shift in the K_i value with incubation time is observed with the N-dealkylation of SKF 525-A but not with SKF 8742-A, metabolite I may be the postulated inhibitory metabolite. Gillette and Sesame (12) showed that SKF 525-A does not inhibit the anaerobic reduction of *p*-nitrobenzoate by microsomal enzymes, but that a metabolite of SKF 525-A, produced under aerobic conditions, does inhibit the anaerobic reduction. The possibility that AEDV or metabolite I may be this metabolite should be considered.

When employed at a substrate concentration of 2×10^{-3} M, SKF 525-A was hydrolyzed by washed hepatic microsomes

at the rate of 0.27 μmoles per gram of liver per hour. The concentration of SKF 525-A used in this study was considerably greater than the K_m concentration (3.6×10^{-5}) for the N-dealkylation of SKF 525-A (1) and probably is a saturating concentration. The maximum velocity for the N-dealkylation reaction is 3.73 μmoles per gram of liver per hour (1). Thus, compared with the dealkylation reaction, hydrolysis is a minor reaction. The acidic hydrolysis product, 2,2-diphenylvaleric acid (SKF 2314) is a relatively poor inhibitor of the microsomal N-demethylation of ethylmorphine. When an ethylmorphine concentration of 8×10^{-4} M was employed, a concentration of 10^{-3} M of SKF 2314 was required to produce a 55% inhibition of the N-dealkylation of ethylmorphine. A concentration of 10^{-4} M produced a 20% inhibition. In view of these considerations the hydrolysis reaction is not thought to play an important role in the inhibition of ethylmorphine metabolism by SKF 525-A.

The application of the peak shift technique to gas–liquid chromatography which was used in the identification of SKF 8742 as a metabolite of SKF 525-A deserves some comment because it is a new procedure that should prove very useful in future studies of drug metabolism. Under a given set of conditions, the retention time is as characteristic of a compound as the melting point. Thus, if one is able to make several derivatives on the column where retention times compare favorably with derivatives of a reference compound, also synthesized on the column, one has identified the unknown compound in accordance with classical procedures of organic chemistry. The quantity or purity of material required to obtain derivatives and their physical constants by conventional means are not demanded by the peak shift technique, and it is thus possible to positively identify microgram quantities of unknown materials in very crude extracts. In the current studies, trapped eluates from the column were used for the peak shift experiments, but in similar studies the peak shift technique was applied to crude extracts with equal success.

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