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FORMATION OF ARTIFACTUAL METABOLITES OF DOXYLAMINE FOLLOWING ACID HYDROLYSIS

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SUMMARY

This study describes the use of gas chromatographic–mass spectrometric, high-performance liquid chromatographic and capillary column gas chromatographic separation techniques in demonstrating the production of several artifactual compounds reported in the literature as metabolites of doxylamine. Rhesus monkey urinary extracts which contained doxylamine and doxylamine metabolites were examined with and without acid hydrolysis. The production of 1-phenyl-1-(2-pyridinyl)ethanol and 1-phenyl-1-(2-pyridinyl)ethylene under acid hydrolysis conditions was demonstrated. These artifactual products were shown to originate from the acid hydrolysis of 2-[1-phenyl-1-(2-pyridinyl)ethoxy]acetic acid and not from doxylamine.

INTRODUCTION

Doxylamine succinate, N,N-dimethyl-2-[1-phenyl-1-(2-pyridinyl)-ethoxy]ethanamine succinate, is a drug used in a therapeutic formulation, Bendectin™ formerly taken by pregnant women as an antinauseant [1]. No evidence was found [2] to suggest that Bendectin was teratogenic in women who took the formulation during pregnancy. However, a recent report concerning the administration of doxylamine succinate to pregnant New Zealand white rabbits [3], although given in large doses and causing lethality in 39% of the animals treated, indicated an increased incidence of deformities in the exposed rabbit embryos. Doxylamine succinate has also been studied for potential genotoxicity using the hepatocyte–DNA repair assay and was shown to induce a weak response at the highest non-toxic dose tested [4]. Also, recent studies in non-human pri-

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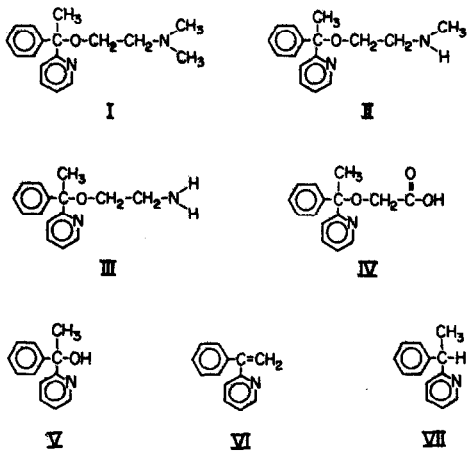

Fig. 1. Structures of doxylamine and its related compounds.

mates dosed with Bendectin during organogenesis have shown an increase in cardiac ventricular septal defects in fetuses examined at 100 days of gestation [5].

The identification of doxylamine (I) and 70% of its urinary metabolites N-desmethyldoxylamine (II), N,N-didesmethyldoxylamine (III), and 2-[1-phenyl-1-(2-pyridinyl)ethoxy]acetic acid (IV) (see Fig. 1) was accomplished by comparing their mass spectra, before and after chemical derivatization, to those of synthetic reference standards [6,7]. Earlier clinical studies [8,9] reported that doxylamine succinate was excreted as the unchanged drug I, the metabolites (II and III), and three other possible hydrolysis products: 1-phenyl-1-(2-pyridinyl)ethanol (V), 1-phenyl-1-(2-pyridinyl)ethylene (VI), and 1-phenyl-1-(2-pyridinyl)ethane (VII). The authors cautioned that these last three products may have been artifactually formed during the acid hydrolysis step of the drug-screening methodology.

The purpose of this report is to describe the analytical methodology used in the pharmacological studies with doxylamine, to present the chemical techniques used in the analyses of the biological samples, and to report on the artifactual production of these three artifactual doxylamine metabolites which were generated from biological samples subjected to the acid hydrolysis procedure.

EXPERIMENTAL

Mass spectrometry

All mass spectrometry (MS) samples were analyzed with a Model 4023 mass spectrometer (Finnigan-MAT) incorporating the standard pulsed positive-ion negative-ion chemical-ionization (CI) electronics and electron-impact chemical-ionization (EI-CI) source. All samples were run in the positive-ion CI mode using 10% ammonia in nitrogen as the reagent gas. The reagent gas was set to an uncorrected nominal pressure of 0.25 Torr and the source temperature was 200°C.

Samples analyzed by fused-silica gas chromatography–mass spectrometry

(GC–MS) were chromatographed via a J & W (Folsom, CA, U.S.A.) DB 5 (30 m×0.25 mm, 0.25 μm film) fused-silica GC column interfaced directly to the mass spectrometer ion source. The GC carrier gas was helium at 1.4 bar. Typically, 1–2 μl of the sample were injected via a Grob-type splitless injector set at 225°C with the oven temperature held at 60°C for 2 min, and then programmed to 240°C at 15°C/min. Samples analyzed by packed column GC–MS were injected into a 1.8 m×2 mm I.D. glass column packed with 1.5% OV 17+1.95% OV 210 on 100–120 mesh Chromosorb W HP. Typically, 1–2 μl of sample were injected into the gas chromatograph and the oven temperature was held at 60°C for 2 min and then programmed to 240°C at 15°C/min. The GC carrier gas was helium set at a flow-rate of 20 ml/min.

Gas chromatography

The GC analyses were conducted with a Tracor Model 560 gas chromatograph equipped with a Tracor Model 702 nitrogen–phosphorus (N–P) detector. A 25 m×0.25 mm J & W fused-silica column (DB 1701) with a 0.25-μm film was used. The helium carrier gas linear flow-rate was 1 ml/min and the nitrogen makeup gas was set at 10 ml/min. The N–P detector was operated at 300°C with the hydrogen and air flow-rates set at 20 ml/min and 120 ml/min, respectively. The temperature program conditions for the GC oven were: initial temperature 200°C with a 6-min hold, and a temperature ramp of 20°C/min with a final temperature of 280°C for 6 min. All injections were 1 μl on-column using a syringe with a 19-cm fused-silica needle. All samples were dissolved in 20% 1-butanol–*n*-dodecane containing 100 ppm of tripelennamine as the internal injection standard [10].

High-performance liquid chromatography

The high-performance liquid chromatographic (HPLC) system was comprised of a Spectra-Physics Model 8700 solvent delivery system utilizing a Rheodyne 7125 injector fitted with a 250-μl loop, a Swagelok guard column (50 mm×4.6 mm I.D.) filled with Supelco LC-CN (50 μm), a Supelco LC-CN (5 μm) analytical column (250 mm×4.6 mm I.D.), a Waters Model 440 ultraviolet detector operated at 254 nm and a Flo-One/Beta (Radiomatic, Tampa, FL, U.S.A.) fully automated, microprocessor/computer-controlled radioactive flow detector for HPLC. An isocratic/gradient mobile phase program (40 min) was employed. Solvent A consisted of methanol–0.01 *M* potassium dihydrogen phosphate (5:95) containing 0.02 *M* triethylamine (TEA), pH 7.3, and solvent B consisted of methanol–0.01 *M* potassium dihydrogen phosphate (95:5) with 0.02 *M* TEA, pH 7.3. After sample injection, initial conditions of A were maintained for 10 min, followed by a 2-min gradient to B with a final hold of 18 min. The column's initial conditions were then regenerated by programming from B to A over 2 min and allowing the column to reequilibrate with solvent A for 8 min. The flow-rate was 1.0 ml/min and the HPLC system pressure was 96–145 bar during the 40-min program run. The Flo-One/Beta radioactivity detector used a 2.5-ml open cell with scintillation cocktail pumped at a flow-rate of 3 ml/min to the detector along with the column effluent (100%), then mixed prior to entering the cell.

Chemical and reagents

Doxylamine succinate was obtained from Richardson Merrell (Cincinnati, OH, U.S.A.), and was used as received after determining a purity of > 99% by HPLC and GC with flame ionization detection (FID) analysis. The [^{14}C]doxylamine was 99% radiochemically pure and was obtained from Southwest Foundation for Research Education (San Antonio, TX, U.S.A.). All organic solvents used were distilled-in-glass quality from Burdick and Jackson Labs. Synthesis standards were prepared or purchased from commercial suppliers for comparison analyses by HPLC, GC, and GC–MS as follows: 1-phenyl-1-(2-pyridinyl)ethylene, 1-phenyl-1-(2-pyridinyl)ethane, and N-desmethyldoxylamine were prepared by C.L. Holder [6] and 1-phenyl-1-(2-pyridinyl)lethanol was prepared by Dr. J. Althaus (NCTR, Jefferson, AR, U.S.A.). Midwest Research Institute (Kansas City, MO, U.S.A.) synthesized both the 2-[1-phenyl-1-(2-pyridinyl)-ethoxy]acetic acid and the N,N-didesmethyldoxylamine.

Dose administration and sample collection

Three adult female rhesus monkeys (*Macaca mulatta*) were used. Food was withheld overnight prior to administration of the drug the following morning. The 85-μCi dosage of [^{14}C]doxylamine succinate was mixed with unlabeled doxylamine succinate and administered intravenously in normal saline for a total dosage of 13.3 mg/kg (twenty times the human equivalent dose). This dose was selected to assure enough mass for physiochemical characterization and dose accountability studies.

Urine samples were collected over dry ice before dose administration and from 0 to 6, 6 to 24, 24 to 48, and 48 to 96 h after dosing and stored at $-20°$C until analyzed.

Acid hydrolysis studies

Two experiments were performed using acid hydrolysis. In the first acid hydrolysis experiment, control and dosed rhesus monkey urine samples (2 ml each) were hydrolyzed under reflux conditions for 30 min with 1:5 ratio of urine to 6 *M* hydrochloric acid in a 125-ml Erlenmeyer flask. These conditions approximate those reported in the literature [8]. The hydrolysate was quantitatively transferred to a 30-ml culture tube and the pH adjusted to 6.0 with approximately 3 ml of 10 *M* potassium hydroxide and the sample extracted with three 10-ml volumes of dichloromethane. The dichloromethane extracts were combined in a 50-ml round-bottom flask and evaporated via water pump aspiration. The contents of the flask were redissolved in methanol for analysis by HPLC. However, for GC–MS analyses, the combined extracts were redissolved in 1 ml of dichloromethane and gaseous diazomethane was introduced to methylate the carboxylic functional group of the acid metabolite of doxylamine. This reaction was terminated after 15 min by evaporating the sample under dry nitrogen at ambient temperature. The extract was then acetylated with 500 μl acetic anhydride (99+%) and 20 μl of pyridine, and after 1 h the acetylation reaction was stopped by evaporating the sample extract under dry nitrogen at ambient temperature. The sample extract was then redissolved in the appropriate amount of dichloromethane for the subsequent fused-silica GC–MS analysis. For comparison, a sec-

ond dosed urine sample was treated as above except that it was not subjected to acid hydrolysis. Also, a solution of the doxylamine and the synthesized standards was prepared for use as a composite standard and derivatized as described above for the rhesus monkey urinary extracts.

In the second acid hydrolysis experiment, triplicate samples of (1) 500 μg of doxylamine and (2) 500 μg of 2-[1-phenyl-1-(2-pyridinyl)ethoxy]acetic acid, the major doxylamine metabolite in the monkey [7] were hydrolyzed in 6 *M* hydrochloric acid under reflux conditions as described above for the control and dosed rhesus monkey urine to ascertain if the acid hydrolysis step would produce the artifacts. The hydrolysates from these procedures were assayed by HPLC, then methylated and acetylated prior to analyses by fused-silica GC–MS.

RESULTS AND DISCUSSION

The artifactual formation of chemical reaction products during chloroform extraction of biological samples has been previously reported for drugs or their metabolites containing amine substituents [11–13]. The avoidance of the formation of these artifacts in biological samples collected in metabolism or pharmacological studies is desirable [14, 15]. In this study, three analytical methods, HPLC, capillary GC with N–P detection (NPD) and GC–MS, were used to demonstrate the formation of the reaction products from a metabolite of doxylamine following a routine acid hydrolysis step, a procedure often used in the analysis of clinical samples.

The HPLC profiles of urine extracts from rhesus monkeys dosed with doxylamine prepared with and without the hydrochloric acid hydrolysis step are shown in Fig. 2. The HPLC of the rhesus monkey urinary sample without acid hydrolysis indicated the sample contained compounds I, II, III, and IV. However, the same rhesus monkey urinary sample subjected to hydrochloric acid hydrolysis indicated the presence of compounds I, II, III, IV, V, and VI. Thus, the HPLC technique which does not require either an extraction step or further chemical derivatization indicated two additional HPLC peaks in the sample after acid hydrolysis.

Rhesus monkey urinary samples (before and after hydrochloric acid hydrolysis) were extracted and derivatized with acetic anhydride and diazomethane [10]. These samples were assayed by fused-silica GC–NPD and the resulting chromatograms are illustrated in Fig. 3. The urinary sample without acid hydrolysis contained compounds I, II, III, and IV as observed with the HPLC method. The urinary sample after acid hydrolysis, however, contained compounds I, II, III, IV, V, and VI. The effectiveness of this methodology for the demonstration of the presence of the two additional compounds is clearly seen in this figure.

Under ammonia CI-MS conditions, the packed column GC–MS analysis of the rhesus monkey urinary extract before the acid hydrolysis step (trace not shown) also demonstrated that the sample contained four compounds I, II, III, and IV, the same compounds which were observed by the HPLC and fused-silica GC–NPD methods. The packed column GC–MS analysis after acid hydrolysis showed six peaks, which were identified as I, II, III, IV, V, and VI. Table I lists the retention

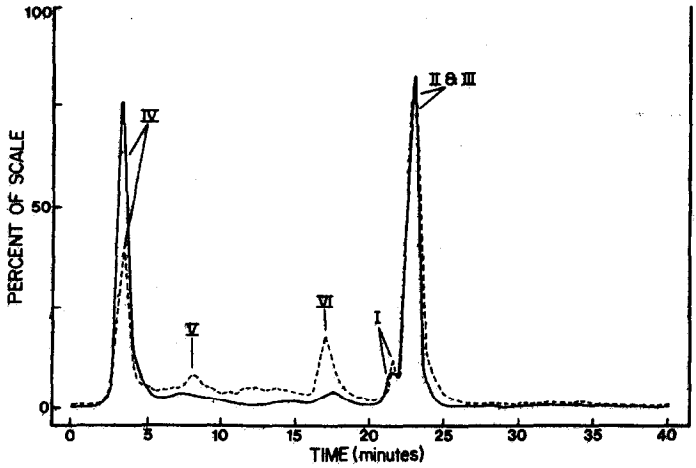

Fig. 2. Typical HPLC gradient profiles of a 24-h urine sample from a rhesus monkey dosed with [^{14}C]doxylamine succinate before and after hydrochloric acid hydrolysis. The solid line represents a 100-μl injection of a 1-ml urinary sample before acid hydrolysis using a Flo-One/Beta radioactivity detector set at a cpm sensitivity scale of 1000. The broken lines (superimposed) illustrate the response from a 100-μl injection of the same urinary sample after acid hydrolysis.

times obtained for doxylamine and its related metabolites and chemical reaction products using the four methods of analysis. For the acid hydrolyzed sample under ammonia CI-MS conditions, the mass spectrum of the parent compound, I, included an m/z 184 ion, the major fragment ion and an $[M+H]^+$ ion at m/z 271 as previously reported for doxylamine [6]. The $[M+H]^+$ ion for acetylated

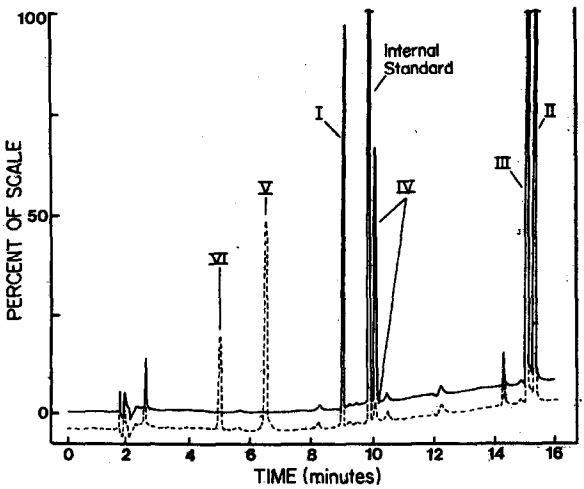

Fig. 3. Typical GC–NPD profiles of a 24-h urine sample from a rhesus monkey dosed with [^{14}C]doxylamine succinate before and after hydrochloric acid hydrolysis. The solid lines represent doxylamine and three related metabolites at an attenuation of 4. The broken lines (superimposed) illustrate the presence of two additional metabolites (compounds V and VI) of doxylamine after hydrochloric acid hydrolysis.

TABLE I

HPLC, FUSED-SILICA GC-NPD AND GC-MS SEPARATION OF REFERENCE STANDARDS FOR DOXYLAMINE AND ITS RELATED CHEMICAL PRODUCTS

Compound No.	Chemical name	Retention time (min)			
		HPLC	GC-NPD	GC-MS*	GC-MS**
I	Doxylamine	21.5	9.1	12.4	12.3
II	N-Desmethyldoxylamine	22.3	15.4***	15.7***	16.3***
III	N,N-Didesmethyldoxylamine	21.8	15.2***	15.6***	16.1***
IV	2-[1-Phenyl-1(2-pyridinyl)ethoxy]acetic acid	3.4	10.2§	12.8§	13.1§
V	1-Phenyl-1-(2-pyridinyl)ethanol	8.5	6.6	10.3	10.6
VI	1-Phenyl-1-(2-pyridinyl)ethylene	17.1	5.2	9.7	9.9
VII	1-Phenyl-1-(2-pyridinyl)ethane	17.5	4.4	9.2	9.2

*Fused-silica capillary column.
**Packed column.
***Compound was acetylated prior to GC analysis.
§Compound was methylated prior to GC analysis.

metabolites II and III was observed at *m/z* 299 and 285, respectively. The packed column GC–MS results for compounds II and III were identical in retention time and MS composition to those obtained for the reference standards of acetylated N-desmethyldoxylamine and N,N-didesmethyldoxylamine, respectively. 2-[Phenyl-1-(2-pyridinyl)ethoxy]acetic acid (IV) after methylation exhibited an ammonia CI mass spectrum with an $[M+H]^+$ ion at *m/z* 272, consistent with the mass spectrum of the reference standard for this compound [7]. The artifactual products from the acid hydrolysis step, 1-phenyl-1-(2-pyridinyl)ethanol (V) and 1-phenyl-1-(2-pyridinyl)ethylene (VI) have $[M+H]^+$ ions at *m/z* 200 and 182, respectively, and their spectra agreed with those previously reported for these compounds [16].

To demonstrate a possible route for the artifactual production of compounds V and VI, 500 μg of compounds I and IV were separately spiked into deionized water, acid-hydrolyzed, and then analyzed by both HPLC and fused-silica GC–MS. The concentration of I (doxylamine) after acid hydrolysis for 30 min was essentially unchanged and V and VI were not observed. However, the fused silica GC–MS profiles, shown in Fig 4, of IV before and after acid hydrolysis clearly demonstrate a production of products from this doxylamine metabolite. Fig. 4A shows the *m/z* 184 trace for the doxylamine metabolite (IV) before acid hydrolysis. The *m/z* 184 ion was selectively traced out because it is the marker ion [6,16] for doxylamine and its metabolites. Fig. 4B shows the reconstructed ion chromatogram (RIC) which is the trace of all the ions monitored for the same analysis as Fig. 4A. Thus, Fig. 4A and B shows the starting material (IV) to be pure. Fig. 4C shows the *m/z* 184 trace for IV after it was subjected to the acid hydrolysis step and Fig. 4D shows the RIC for this same analysis. The GC peaks for the products formed from the acid hydrolysis of IV are evident in Fig. 4C and D.

The following peak assignments for Fig. 4C and D are: peak 1 (labeled VII),

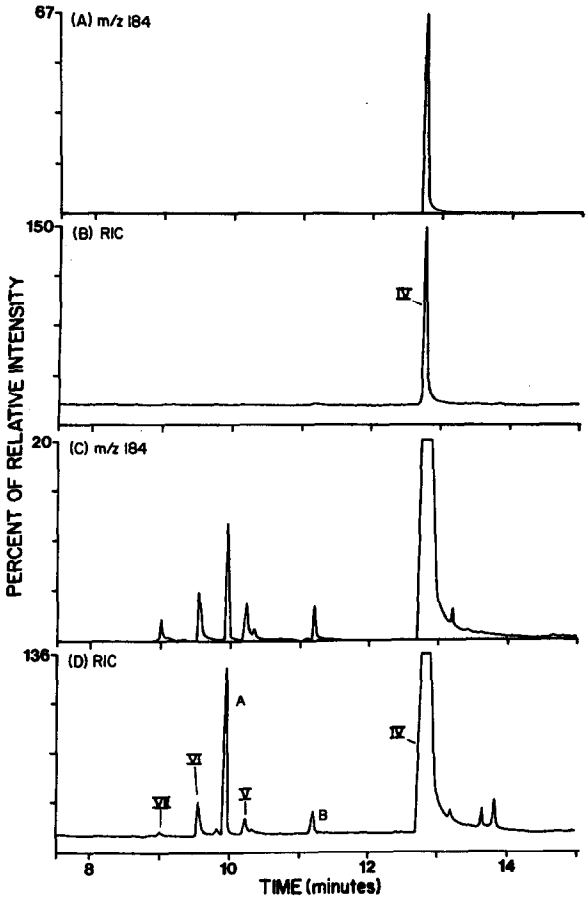

Fig. 4. Ammonia CI fused-silica GC–MS chromatograms showing: (A) *m/z* 184 and (B) the reconstructed ion chromatogram (RIC) for a sample of compound IV before acid hydrolysis and (C) *m/z* 184 and (D) RIC for a sample of compound IV after acid hydrolysis. Peaks A and B are tentatively identified as 1-phenyl-1-(2-pyridinyl)methylethyl ether and 1-phenyl-1-(2-pyridinyl)ethylethanol ether, respectively.

the $[M+H]^+$ ion was at *m/z* 184 and the compound was identified as VII; peak 2 (labeled VI), the $[M+H]^+$ ion was at *m/z* 182 and the product was identified as VI; peak 3 (labeled A), the $[M+H]^+$ ion was at *m/z* 214 and the compound was tentatively identified as 1-phenyl-1-(2-pyridinyl)methylethyl ether; peak 4 (labeled V), the $[M+H]^+$ ion was at *m/z* 200 and the product was identified as V; and peak 5 (labeled B), the $[M+H]^+$ ion was at *m/z* 244 and the compound was tentatively identified as the 1-phenyl-1-(2-pyridinyl)ethylethanol ether. It should be noted that while compound VI has a molecular weight of 181, under ammonia CI-MS conditions the mass spectrum of this compound includes a major peak at *m/z* 184 presumably due to a reduction process similar to that described by Korfmacher et al. [16] for doxylamine.

These products, except for A and B for which standards were unavailable, were determined and quantitated by HPLC as listed in Table II. The chemical products

TABLE II

PERCENTAGE OF EACH CHEMICAL PRODUCT FORMED AFTER ACID HYDROLYSIS OF 2-[1-PHENYL-1-(2-PYRIDINYL)ETHOXY]ACETIC ACID METABOLITE OF DOXYLAMINE

The percentage change was quantitated by HPLC.

Compound No.	Chemical name	Percent change
IV	2-[1-Phenyl-1-(2-pyridinyl)ethoxy]acetic acid	−40
V	1-Phenyl-1-(2-pyridinyl)ethanol	+10
VI	1-Phenyl-1-(2-pyridinyl)ethylene	+25
VII	1-Phenyl-1-(2-pyridinyl)ethane	+ 5

produced by acid hydrolysis which have been previously identified as doxylamine metabolites by other workers are: 1-phenyl-1-(2-pyridinyl)ethanol (V), 1-phenyl-1-(2-pyridinyl)ethylene (VI), and 1-phenyl-1-(2-pyridinyl)ethane, (VII). In addition, VII was observed as a doxylamine metabolite in body fluid from cases of acute poisoning [17]. We believe that these compounds (V, VI and VII) may not be metabolites but may be artifactually produced when an acid clean-up is used.

In conclusion, we have shown that the acid hydrolysis clean-up step produced doxylamine metabolites not observed in biological samples without this clean-up step, and that these are apparently formed from the 2-[1-phenyl-1-(2-pyridinyl)ethoxy]acetic acid metabolite of doxylamine. Therefore, we caution researchers involved in the clean-up and analysis of doxylamine metabolites or related compounds to avoid using the acid hydrolysis step in their procedure.

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