Separation and Detection of Doxylamine and Its Rhesus Monkey Urinary Metabolites by High Resolution Gas Chromatography Utilizing Nitrogen/Phosphorus Detection

L. G. Rushing*, A. B. Gosnell[1], **C. L. Holder, W. A. Korfmacher,** and **W. Slikker, Jr.**
Department of Health and Human Services, Food and Drug Administration, National Center for Toxicological Research, Jefferson, Arkansas 72079, USA.

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High resolution gas chromatography
Nitrogen/phosphorus detector
Doxylamine metabolites
Antihistamines
Rhesus monkey

Summary

A high resolution gas chromatographic method is described for the separation of rhesus monkey urinary metabolites of doxylamine with the use of on-column injection, a fused silica column (DB-1701), and nitrogen/phosphorus detection. An ancillary separation of doxylamine and six other antihistamines is also presented.

1 Introduction

Doxylamine, *N,N*-dimethyl-2[1-phenyl-1-(2-pyridinyl)ethoxy]ethanamine (VII), is an antihistamine found in numerous common pharmaceutical preparations for the control of migraines, allergies, and motion sickness. It is also used in over-the-counter sleep aids and has been used in the past to control nausea of pregnancy. Production in the United States in 1980 was at the rate of 165 metric tons for the formulation of these pharmaceutical products [1]. Studies with rabbits and rats and in other studies with pregnant women indicated no anomalous fetal development after exposure to Bendectin™ (a doxylamine therapeutic formulation) [1-3]. *Hendrickx et al.* [4] recently reported that Bendectin™ administration to monkeys or baboons early in pregnancy resulted in an increased incidence of ventricular septal defects when the offsprings were observed prenatally. The absence of defects in monkeys at term, however, prompted *Hendrickx et al.* [4] to suggest that a delay in closure of the ventricular septum was the only observable effect of Bendectin™ exposure.

Little was known concerning the metabolic fate of doxylamine in humans prior to 1981. In that year, *Gielsdorf* and *Schubert* [5] reported the identification of six human urinary products (**Table 1**): the unchanged drug (VII), 1-phenyl-1-(2-pyridinyl)ethane (I), 1-phenyl-1-(2-pyridinyl)-ethene (II), 1-phenyl-1-(2-pyridinyl)ethanol (III), didesmethyldoxylamine (V), desmethyldoxylamine (VI), and an additional unidentified minor product. These metabolites were separated by thin layer chromatography, the spots scraped, derivatized as necessary, and identified by mass spectrometry. They reported, however, that I and II may have been artifactually produced. *Koppel* and *Tenczer* [6], furthermore, described a metabolic pathway to I by the tentative identification of a previously unknown metabolite, 2-[1-phenyl-1-(2-pyridinyl)ethoxyl]acetic acid (IV).

This paper describes a rapid chromatographic separation (16 min) for compounds I, II, III, IV, V, VI, and VII by capillary gas chromatography developed during our investigation of doxylamine and its rhesus monkey urinary metabolites. Our chromatographic separation has the advantage of high resolution capillary retention time specificity combined with the specificity of a nitrogen/phosphorus rubidium bead detector (N/P). Because the N/P detector has a selective specificity for nitrogen over hydrocarbons, it has become a valuable tool in the analysis of nitrogen-containing drugs. Also illustrated is the ancillary chromatographic separation of seven antihistamines by high resolution gas chromatography with N/P detection.

2 Experimental

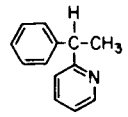
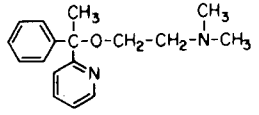
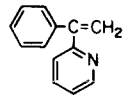
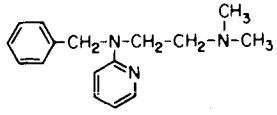
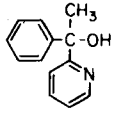
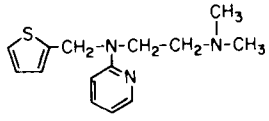
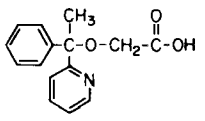
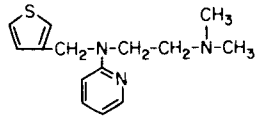
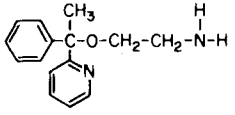
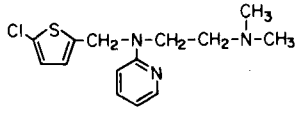
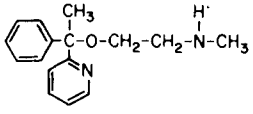
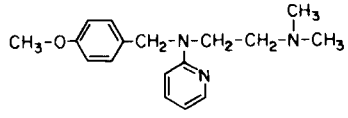
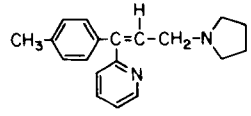
2.1 Chemicals and Supplies

Antihistamines were obtained from the following sources: doxylamine succinate (Richardson Merrell, Inc., Cincinnati, Ohio); tripelennamine hydrochloride (Ciba Geigy, Summit, NJ); methapyrilene hydrochloride (Abbott Lab., North Chicago, IL); thenyldiamine hydrochloride (Sterling Organics, Dudley, Cramlington, Northumberland, England); chlorothen citrate (NCTR, *Dr. J. Althaus,* Jefferson, AR); pyrilamine maleate (Hexagon Lab Inc., Bronx, NY) and

[1] Henderson State University, Arkadelphia, AR 71923. Faculty Appointment at the NCTR

Table 1

Antihistamines, doxylamine metabolites and related compounds

Structure	Name	Retention Time	Structure	Name	Retention Time
	I 1-phenyl-1-(2-pyridinyl) ethane	4.36		VII doxylamine N,N-dimethyl-2-[1-phenyl-1-(2-pyridinyl) ethoxy] ethanamine	9.13
	II 1-phenyl-1-(2-pyridinyl) ethene	5.15		VIII tripelennamine 2-[benzyl (2-dimethylamino-ethyl) amino] pyridine	10.00
	III 1-phenyl-1-(2-pyridinyl) ethanol	6.63		IX methapyrilene 2-[(2-dimethylaminoethyl)-2-thenylamino] pyridine	10.18
	IV 2-[1-phenyl-1-(2-pyridinyl) ethoxy] acetic acid	10.18[a]		X thenyldiamine 2-[(2-dimethylaminoethyl)-3-thenylamino] pyridine	10.34
	V didesmethyldoxylamine 2-[1-phenyl-1-(2-pyridinyl) ethoxy] ethanamine	15.22[b]		XI chlorothen 2-[(5-chloro-2-thenyl)(-2-dimethylaminoethyl) amino] pyridine	11.81
	VI desmethyldoxylamine N-methyl-2-[1-phenyl-1-(2-pyridinyl) ethoxy] ethanamine	15.43[b]		XII pyrilamine 2-[(2-dimethylaminoethyl) (p-methoxybenzyl) amino] pyridine	12.74
				XIII triprolidine trans-2-[3-(1-pyrrolidinyl)-1-p-tolylpropenyl] pyridine	12.74

a) t_R of methylated product

b) t_R of acetylated product

triprolidine hydrochloride (Chemical Dynamics, South Plainfield, NJ). The ^{14}C-doxylamine was > 99% radiochemically pure and was obtained from Southwest Foundation for Research and Education (San Antonio, TX). The identities and purities of these antihistamines were verified by HPLC, mass spectrometry, and/or nuclear magnetic resonance.

Synthesis standards were prepared or obtained for comparison as follows: 1-phenyl-1-(2-pyridinyl)ethane (I), 1-phenyl-1-(2-pyridinyl)ethene (II) and desmethyldoxylamine (VI) were prepared by *C.L. Holder* (NCTR, Jefferson, AR) and the 1-phenyl-1-(2-pyridinyl)ethanol (III) was prepared by *Dr. J. Althaus* (NCTR, Jefferson, AR). The synthesis of compounds I, II, and III were by the following scheme. Compound III was prepared from 2-benzopyridine and methyl magnesium bromide and was purified by vacuum distillation. Product III was acidified with 12N HCl and heated at 85°C for 5 hours to produce II which was then reduced with palladium powder in 88% formic acid to yield compound I. The preparation of VI is described elsewhere [3]. Midwest Research Institute (Kansas City, MO) synthesized both the 2-[1-phenyl-1-(2-pyridinyl)ethoxy]acetic acid (IV) and the didesmethyldoxylamine (V) whose structures were verified as above. The organic solvents used were distilled-in-glass quality from Burdick and Jackson Laboratories, Inc. (Muskegon, MI) except for 1-butanol (J.T. Baker, Phillipsburg, NJ) and *n*-dodecane (Aldrich Chemical Company, Milwaukee, WI).

2.2 Sample Preparation

Rhesus monkeys were dosed intravenously with 85 μCi of ^{14}C-doxylamine succinate (32.5 mCi/mmol) mixed with nonlabeled doxylamine in normal saline for a total dose of 13.3 mg/kg. The ^{14}C-labeled compound was used to trace the metabolites during isolation and dose accountability studies performed by other investigators [7]. The rhesus monkey urine was collected over dry ice and stored at −20°C until analyzed. Control and dosed rhesus monkey urine samples were acid hydrolyzed, extracted, acetylated, methylated and dissolved in a 20% 1-butanol/*n*-dodecane solution which contained 100 μg/mL of tripelennamine as an internal standard for capillary GC analysis. The acid hydrolysis, extraction and derivatization procedures have been previously described [3]. In brief, rhesus monkey urine (2 mL) was hydrolyzed under reflux conditions for 30 min with a 1:5 ratio of urine to 6N hydrochloric acid. The hydrolyzate was quantitatively transferred by disposable glass pipette to a culture tube, the pH adjusted to six with 10N KOH and the sample extracted with 3 × 10 mL volumes of dichloromethane, The extracts were combined, evaporated, and acetylated. Both didesmethyldoxylamine (V) and desmethyldoxylamine (VI) were acetylated with 500 μL acetic anhydride (99+%) and 20 μL of pyridine. After one hour, the reaction mixture was evaporated under dry nitrogen at ambient temperature and redissolved in 1 mL dichloromethane. Gaseous diazomethane was then introduced to methylate the carboxylic acid functional group of component IV. The sample was then evaporated, redissolved in 2 mL of dichloromethane and an aliquot of 200 μL removed, evaporated, and redissolved in 200 μL of 20% 1-butanol/*n*-dodecane containing the internal standard in preparation for the subsequent gas chromatographic analysis.

Doxylamine (VII) and the synthesized standards were prepared as composite standards by combining aliquots so as to have concentrations of each compound at 100, 50, 30, 20, and 10 μg/mL where possible. The admixtures were derivatized and prepared for analysis as described above. A solution of the seven antihistamines was also prepared by combining aliquots of the individual antihistamines to yield a solution having 10 μg/mL of each compound. The internal standard (tripelennamine, 100 μg/mL) was not required for this sample since it was one of the components of the antihistamine admixture.

2.3 Gas Chromatography

The analyses were conducted on a Tracor (Austin, TX) model 560 gas chromatograph equipped with a Tracor model 702 N/P detector. A J&W (Folsom, CA) fused silica capillary on-column injector was custom installed between the two standard injection ports on the gas chromatograph. The injection ports were turned off and allowed to cool completely before operation of the on-column injector. A bonded J&W DB-1701 fused silica 25 m × 0.25 mm column with a 0.25 micron film thickness was installed in the gas chromatograph. The helium carrier linear flow rate was approximately 23 cm/s and the nitrogen make up gas flow rate was approximately 10 mL/min. The N/P detector was operated at 300°C with a hydrogen and air flow of 2.0 and 120 mL/min, respectively. The polarizing voltage of the N/P detector was set to the high position (-150V). Column temperature program conditions were as follows: initial temperature 200°C with a six min initial hold, temperature ramp of 20°/min, and a final temperature of 280°C with a final hold of six min. Samples for injection were dissolved in 20% 1-butanol/*n*-dodecane containing 100 μg/mL tripelennamine as an internal standard. The antihistamines were chromatographed in the free base form and all injections were 1 μL on-column via a syringe with a 19 cm fused silica needle. The injector was raised to approximately one half of its fully extended length during injection, the sample injected, the syringe was then removed and the injector plunged to its down position. The resulting chromatographs were recorded on a Shimadzu (Columbia, MD) model C-R3A recording integrator.

2.4 Mass Spectrometry

Packed column GC-mass spectrometry (MS) was used for confirmation of the identities of the metabolites in the rhesus monkey urine extract. The analyses were perform-

ed by chemical ionization mass spectrometry (CIMS) using 10% NH_3 in nitrogen as the reagent gas. All CIMS experiments were performed with a Finnigan-MAT 4023 mass spectrometer system incorporating the standard PPINICI (Pulsed Positive Ion Negative Ion Chemical Ionization) electronics and an electron impact/chemical ionization (EI/CI) source. The GC carrier gas was helium set at a flow rate of 20 cm^3/min. The GC column was a 6 ft × 2 mm i.d., glass column packed with 1.5% OV17+1.95% OV210 on 100/120 mesh Chromosorb WHP. Typically, 1-2 μL of sample were injected and the oven temperature was held at 60°C for 2 min and then programmed to 240°C at 15°/min. All analyses were in the positive ion mode. The reagent gas was used as the make-up gas and was set to an uncorrected nominal source pressure of 0.25 Torr. The source temperature was 200°C. The MS was scanned from m/z 100 to m/z 750 at a rate of two seconds per scan.

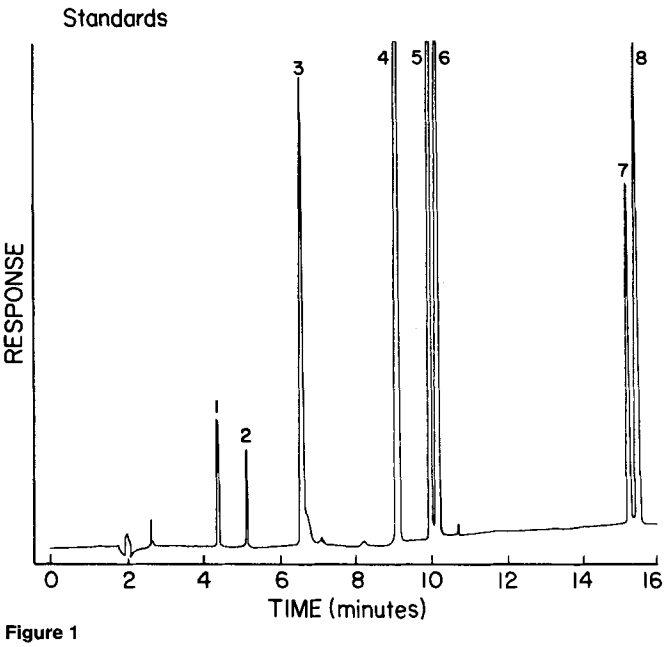


Figure 1

GC/NP chromatogram of standards: (1) I ≅ 8 ng, (2) II ≅ 7 ng, (3) III = 50 ng, (4) VII = 50 ng, (5) VIII = 100 ng, Int. Std., (6) IV, (7) V = 27 ng and (8) VI = 50 ng; V and VI acetylated.

3 Results and Discussion

Table 1 shows the chemical structures and names for the identified rhesus monkey urinary metabolites of doxylamine, some related compounds, and six other antihistamines of interest. All of the rhesus monkey urinary metabolites in Table 1 contain at least one nitrogen, some two, and most of the antihistamines have three nitrogens. Thus, the selection of a N/P detector for these compounds is a logical choice not only because of the sensitivity of the N/P detector for nitrogen containing compounds but also because it is selectively blind to many interferences that could arise from the extraction of the monkey urine matrix. Inspection of Table 1 indicates that many of these chemicals are very similar in structure and would be difficult to resolve by conventional packed column gas chromatography. For example, V and VI differ only by the substitution of a methyl for a hydrogen and pairs IX and X differ only by positional attachment (*i.e.*, 2 or 3) in the thenylamino group. Table 1 also includes the high resolution gas chromatographic retention times (t_R's) for these compounds and shows that despite their chemical similarity they are separated by the DB-1701 capillary phase. Furthermore, the chromatographic separation is illustrated by **Figure 1** for compounds I through VIII. The separation and peak shape for all eight compounds were excellent with the exception of component III which tailed. Most of the compounds are baseline separated. The excellent separation of this eight-component admixture in only 16 min with good chromatography was achieved by injecting the sample on-column employing the "solvent effect" [8,9] produced by the *n*-dodecane. The 1-butanol was added to increase sample solubility.

Tripelennamine (VIII) was selected as the internal injection standard because it responded well on the N/P detector (*i.e.*, 3 nitrogens), was approximately in the center of the chromatogram and did not interfere or coelute with any of the rhesus monkey metabolites. The internal standard was added to the sample just before injection into the GC to serve as a relative retention time standard for identification by an integrator of the other peaks in the chromatographic run and to compensate for the small variation in the nominal 1 μL volume of sample injected into the on-column injector.

Components III, VI, VII, and VIII were prepared from weighed standards. Components I, II, IV, and V were prepared only as qualitative standards because there were insufficient amounts of these materials available for the preparation of quantitative standards; however, quantitation of V was accomplished by comparison of its peak area to the peak area of VI. Compound V and VI are very similar in structure in that both contain a nitrogen atom in the pyridinyl group and in the ethanamine group of the molecule. Thus, their responses on a N/P detector should be comparable. Similarly, peaks I and II could have been quantitated against III. However, because peaks I and II were small and the calibration line for peak III was offset from the origin (possibly because of its peak shape), peaks I and II were estimated by gas chromatographic flame ionization analysis by comparison of their areas to the area of a known amount of the internal standard. The GC-FID temperature and column conditions were identical to those used for the GC-NP analyses. These quantitative and qualitative standards were combined as composite standards which were derivatized, assayed, and found to yield linear responses on the N/P detector. The correlation coefficients (r) for compounds III, V, VI, and VII were 1.00 and for I and II

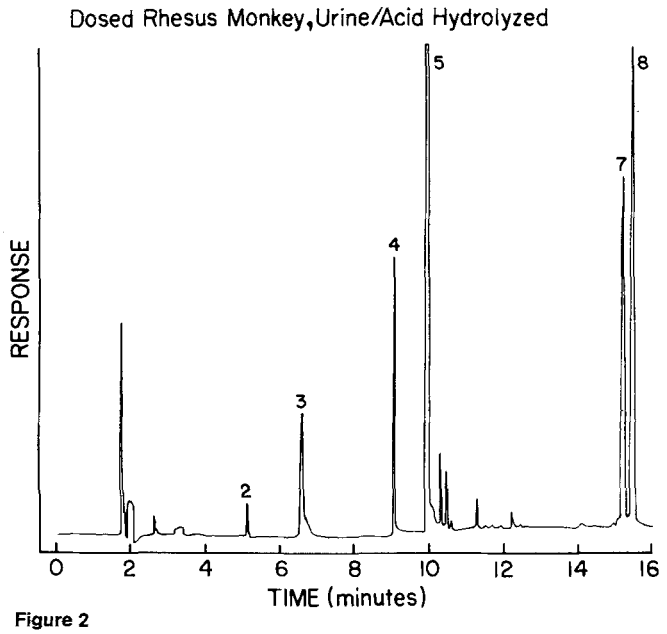


Figure 2

GC/NP chromatogram of the acid hydrolyzed urine from a doxylamine dosed rhesus monkey: (2) II ≅ 3 ng, (3) III = 28 ng, (4) VII = 8 ng, (5) VIII = 100 ng Int. Std. (7) V = 28 ng and (8) VI = 40 ng. Peaks 2 and 3 are possible artifacts of the acid hydrolysis step of the procedure. V and VI acetylated.

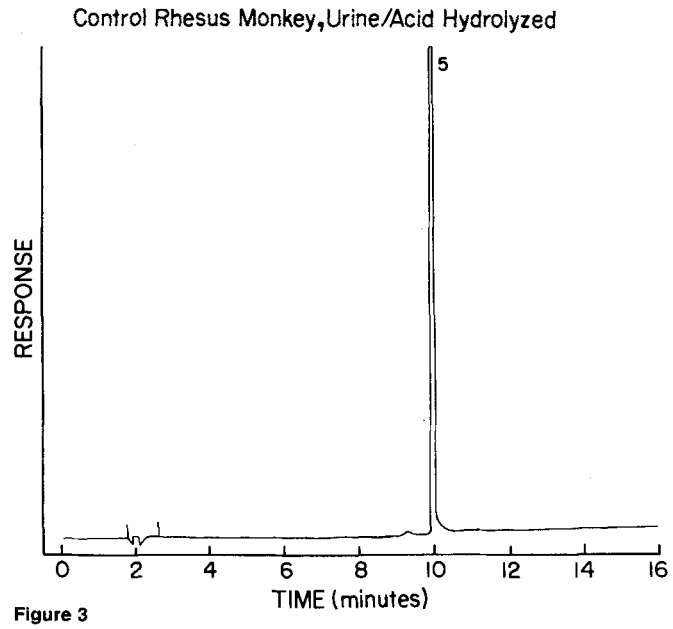


Figure 3

GC/NP chromatogram of the acid hydrolyzed control urine from a rhesus monkey: (5) VIII = 100 ng Int. Std.

0.99 and 0.98, respectively. Only component IV significantly deviated from a linear response. This has been attributed to the incomplete methylation of the acid during the derivatization process. This difficulty, however, could possibly be rectified by reversing the order of the acetylation-methylation derivatization reactions. If the investigator was interested principally in component IV, the extraction from the rhesus monkey urine should be performed at a lower pH favoring a greater recovery of the acid. All of the antihistamines have partition values (p-values) greater than 0.7 in dichloromethane *vs* pH6. Therefore, approximately 70% was in the organic phase *vs* the aqueous phase. Three (3) extractions with dichloromethane should be sufficient for their recovery. At higher pH's, for example pH7, the p-values are 1.00; however, the inverse is true for the acid metabolite (IV). Therefore, an intermediate pH was required to extract both the amine metabolites and the acid metabolite (IV).

Figure 2 is a chromatogram of a dosed rhesus monkey urine sample. The urine was acid hydrolyzed by the method of *Gielsdorf* and *Schubert* [5]. Essentially the same elimination products as they reported (*i.e.*, II, III, V, VI, and VII) were observed in the single 16 min high resolution gas chromatographic separation except for product I. It is possible that peaks II and III are artifacts produced by the acid hydrolysis step since these components were not observed with a neutral pH extraction procedure [3]. **Figure 3** is a chromatogram of the corresponding acid hydrolyzed control rhesus monkey urine and displays only the added internal standard peak (VIII). Figures 2 and 3 illustrate that these doxylamine rhesus monkey urinary metabolites are separated by high resolution gas chromatographic analysis with little interference from the urine matrix itself.

The identities of the metabolites were confirmed by GC-MS using chemical ionization mass spectrometry (CIMS) for the analysis. **Figure 4** shows the mass spectra of III and acetylated V. Under NH_3 CI conditions, as shown in Figure 4(a), III provides an $[M+H]^+$ ion at m/z 200 as the base peak and a major fragment ion at m/z 184 due to the $[M+H-O]^+$ as described recently by *Korfmacher et al.* [10]. The fragment ion at m/z 184 is characteristic of doxylamine related compounds analyzed under NH_3 CI conditions [3,10]. The smaller fragmentation at m/z 122 is due to the $[M+H-C_6H_6]^+$ ion. The NH_3 CI mass spectrum of acetylated V shown in Figure 4(b) displays the $[M+H]^+$ ion at m/z 285 and the characteristic m/z 184 fragment ion as the base peak. The minor fragment peak at m/z 104 is presumably due to the $[M+H-(C-C_6H_5-C_5H_4N-CH_2)]^+$ ion.

The NH_3 CI mass spectrum of II provided the $[M+H]^+$ ion at m/z 182 as the base peak plus the formation of the m/z 184 ion as has been described previously [10]. The NH_3 CI mass spectrum of acetylated VI provided an $[M+H]^+$ ion at m/z 299 plus the m/z 184 fragment ion as the base peak. A minor fragment ion at m/z 118 was probably due to the $[M+H-(C-C_6H_5-C_5H_4N-CH_2)]^+$ ion. Thus, the NH_3CI mass spectral results for II and acetylated VI agreed well with previous reports of the NH_3CIMS analyses of these compounds [3,10].

Ancillary information is presented in **Figure 5** for the chromatographic separation of doxylamine and six additional antihistamines. Five of the seven compounds are baseline

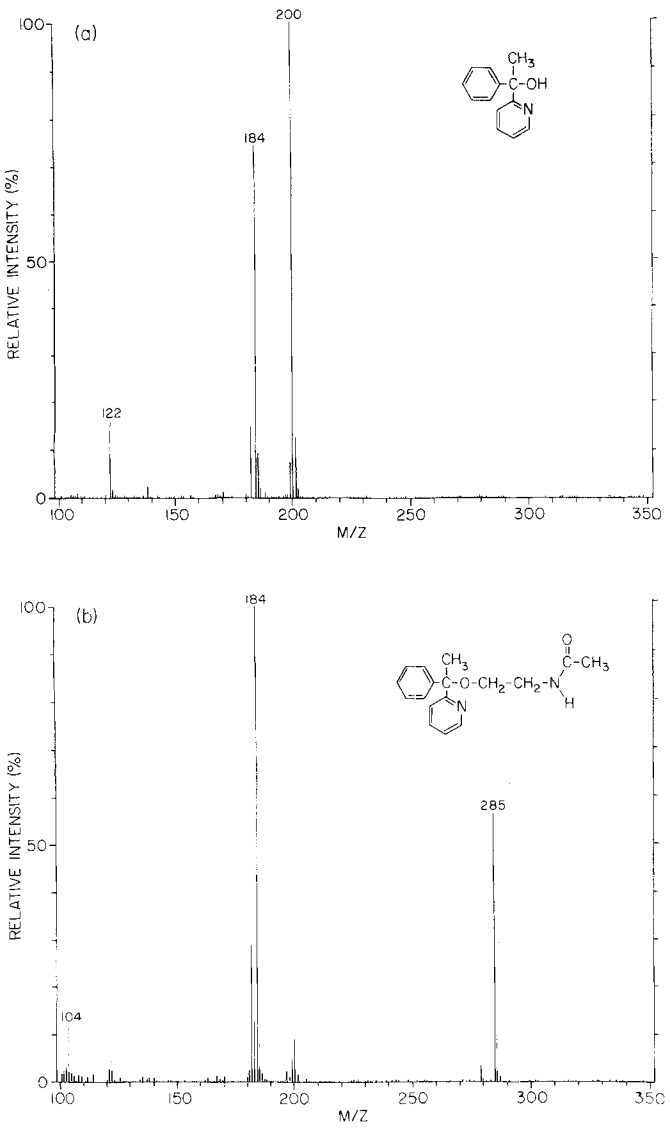


Figure 4

Ammonia CI mass spectrum of (a) metabolite III and (b) acetylated metabolite V.

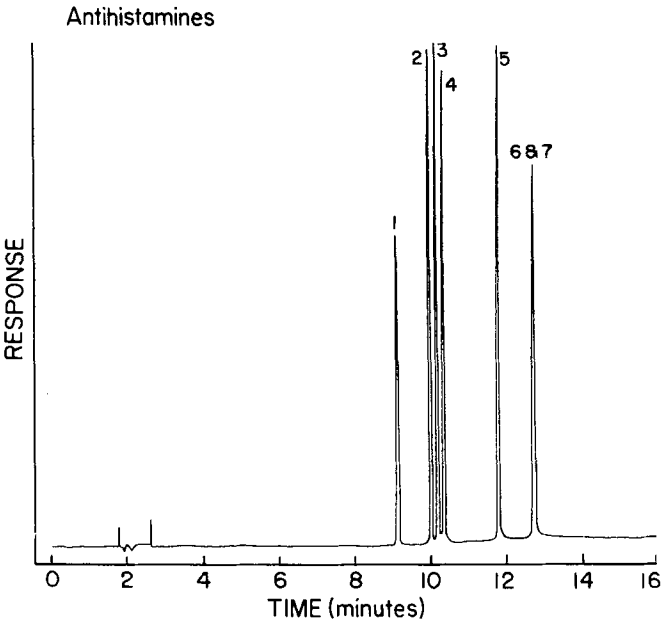


Figure 5

GC/NP chromatogram of the separation of seven antihistamines (10 ng each): (1) VII, (2) VIII, (3) IX, (4) X, (5) XI, and (6&7) XII & XIII.

or nearly baseline resolved. Pyrilamine and triprolidine coelute as one peak with no indication of any separation on a DB-1701 phase. This pair can be easily baseline resolved on a DB-1 phase. However, the closely spaced nearly baseline separated antihistamines (VIII, IX, and X) are then shoved together, Therefore, the overall separation of these antihistamines was superior on the DB-1701 phase.

In conclusion, this paper presents an on-column solvent-effect high resolution gas chromatographic separation for doxylamine and its metabolites and for doxylamine and six other antihistamines. In general, this technique could probably be expanded to include the separation of the metabolites of any of these antihistamines.

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