GC/MS Characterization of Urinary Metabolites of Doxylamine Succinate: Identification of the Aglycones Formed from Intestinal Microflora Metabolism of the Polar Glucuronide Metabolites

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Summary

This study describes the use of high performance liquid chromatography (HPLC) and capillary gas chromatography/mass spectrometry (GC/MS) in the characterization of polar glucuronide conjugates of doxylamine and their subsequent aglycones following deconjugation. Rat urinary extracts which contained doxylamine and both nonconjugated and conjugated doxylamine metabolites, were examined by HPLC before and after incubation with rat intestinal microflora. The subsequent deconjugated urinary metabolites and the nonconjugated products remaining in the urinary extracts were then isolated, acetylated, and assayed by GC/MS. Incubation with the intestinal microflora indicated that anaerobic bacteria were capable of effecting hydrolytic cleavage of these polar *O*-glucuronide metabolites of doxylamine and its demethylated products to their subsequent aglycones. GC/MS analysis was performed using a fused silica DB-5 GC column and was utilized for the identification of these deconjugated metabolites.

1 Introduction

The antihistamine doxylamine succinate is a component in the previously used drug, Bendectin [1–5]. Numerous studies have investigated the health effects and metabolic activity of this compound [6–15]. Early analytical studies of the urinary metabolites of doxylamine succinate and other related antihistamine drugs relied on colorimetric analysis, thin layer chromatography, or infrared measurement [16–18]. Recently, chemical ionization mass spectrometry (CIMS), fast atom bombardment mass spectrometry (FAB/MS), and thermospray/mass spectrometry (TSP/MS) have been used to identify urinary metabolites of doxylamine and related compounds [11,14,19–23]. This paper describes a gradient high performance liquid chromatography (HPLC) method and a capillary gas chromatography (GC/MS) separation and identification of two aglycones from *O*-glucuronide metabolites of doxylamine found in the urine of experimental animals treated with doxylamine succinate. This is the first report of a GC/MS separation for the analysis of the acetylated aglycones of hydroxydoxylamine and hydroxydesmethyldoxylamine. To assess the role of intestinal microflora in the deconjugation of antihistamines, urinary metabolites of doxylamine were incubated with rat intestinal microflora and the corresponding aglycones were isolated, derivatized (acetylated), and analyzed by high resolution capillary GC/MS.

2 Experimental

2.1 Chemicals

Doxylamine succinate, *N,N*-dimethyl-2-[1-phenyl-1-(2-pyridinyl)ethoxy]-ethanamine succinate (Richardson & Merrell, Inc., 99+% pure) and ^{14}C-labeled doxylamine succinate (Southwest Foundation for Research and Education, 98.2% pure) were assayed by HPLC and thin layer chromatography (TLC) using radiochemical detection. The structural identity of the two doxylamine succinate standards (unlabeled or ^{14}C-labeled) were confirmed by mass spectrometry [12].

All organic solvents used in the HPLC analyses were UV grade and all reagents were CP grade.

2.2 Derivatization Techniques

2.2.1 Acetylation

The acetylation of the samples was achieved using acetic anhydride in pyridine. The following procedure was used. The samples in methanol were evaporated to dryness, 50 μL of pyridine and 250 μL of acetic anhydride were added to the 1 mL vial, the contents in the tube were sealed with a Teflon-lined screw cap and allowed to react overnight (16 h). The contents of the vial were then evaporated to dryness under nitrogen at ambient temperature for subsequent GC/MS analysis after addition of the appropriate volume of hexane or ethyl acetate.

2.3 Analytical Methods

2.3.1. GC/MS

The samples were analyzed with a model 4023 mass spectrometer (Finnigan-MAT, San Jose, CA) incorporating the standard pulsed positive ion negative ion chemical ionization electronics and EI/CI source. The samples were introduced via a 30-m DB-5 J&W (Folsom, CA) fused silica GC column (0.25 mm i.d., 0.25 μm film thickness) and analyzed by ammonia chemical ionization mass spectrometry (CIMS). The GC carrier gas was helium set at 20 psi. Typically, 1 μL of the sample was injected via an SGE (Austin, TX) OCI-3 on-column injector. Except where indicated, the oven temperature was held at 60 °C for 2 min after injection of the sample and then programmed to 240°C at 15°/min. The 10% ammonia in nitrogen reagent gas was set to an uncorrected nominal source pressure of 0.25 torr and the source temperature was 200 °C.

2.3.2 HPLC

The gradient HPLC system used consisted of a Spectra Physics 8700 solvent delivery system, a Rheodyne 7125 injector fitted with a 250 μL sample loop, a Swagelok guard column (50 mm × 4.6 mm i.d.) filled with Supelco LC-CN (50 μm), an analytical column (250 mm × 4.6 mm i.d.) also filled with Supelco LC-CN (5 micron), and a Waters 440 ultraviolet detector operated at 254 nm along with a Flo-one Beta fully automated, microprocessor/computer controlled radioactivity detector set in series. The mobile phase used in the linear gradient program was: A (5:95) methanol/0.005 M potassium phosphate buffer containing 0.02 M triethylamine (TEA) with the pH adjusted to 7.8 with phosphoric acid, and B (95:5) methanol/0.005 M potassium phosphate buffer containing 0.02 M TEA (pH 7.8) [13].

3 Results and Discussion

High performance liquid chromatography (HPLC) was used to separate and isolate the polar glucuronide metabolites of doxylamine from rat urine. The glucuronide metabolites elute during the 0–20 min segment of the HPLC chromatographic run, and this fraction contains the separated *O*-glucuronide metabolites of desmethyldoxylamine (**1**) and doxylamine (**2**) [13]. These two conjugated metabolites were then collected at the retention times of 10.80 min for compound **1** and 16.60 min for compound **2** and their structure confirmed by MS and NMR [23]. The two *O*-glucuronide metabolites in the urine sample were then deconjugated with rat intestinal microflora to their resulting aglycones whose HPLC retention times were 24.0 min (hydroxydoxylamine) and 34.2 min (hydroxydesmethyldoxylamine), respectively. Similar results were also found with these two *O*-glucuronide metabolites when human and mouse intestinal microflora were used [13]. The two aglycone peaks were isolated and acetylated prior to analysis by GC/MS. **Figure 1** shows the rat urine before and after microbial deconjugation and illustrates the separation of the *O*-glucuronide metabolites (peaks 1 and 2) and the nonconjugated metabolites (peaks 3 and 4) of doxylamine. Figure 1B demonstrates that after 24 h of incubation with rat intestinal microflora, the peaks corresponding to the conjugated metabolites were diminished and peaks # 5 and # 6 were left. After HPLC isolation, peak # 5 was acetyated and analyzed via GC/MS and found to contain a mixture of doxylamine and acetylated hydroxydoxylamine. After HPLC isolation, peak # 6 was acetylated and analyzed via GC/MS and shown to contain acetylated desmethyldoxylamine and acetylated hydroxydesmethyldoxylamine.

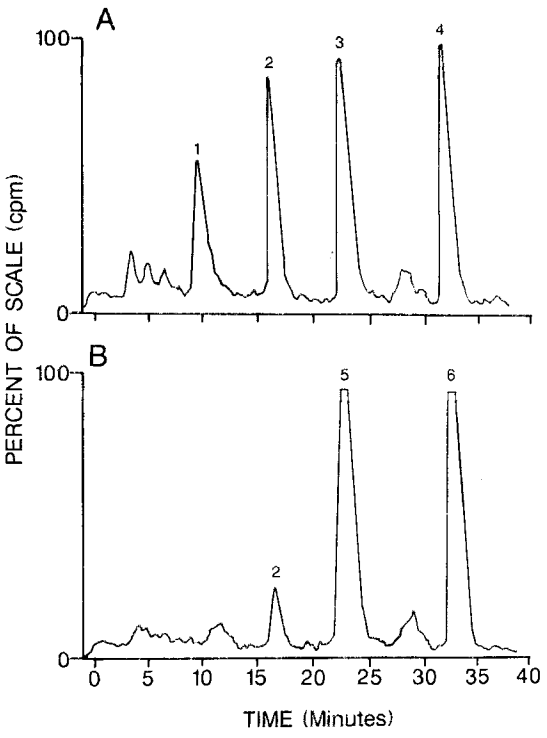

Figure 1

The HPLC chromatogram of the rat urine before (1A), and after (1B) intestinal microflora incubation.

Figure 2 illustrates the GC/mass chromatogram obtained as outlined above for HPLC fraction # 5 (shown in Figure 1B). The main GC peak **a** (retention time *ca.* 12.5 min) shown in Figure 2, produced an ammonia CI mass spectrum with a base peak at m/z 184 and major peak at m/z 271. This mass spectrum corresponds well with the published ammonia CI mass spectrum for doxylamine in which m/z 271 is the $[MH]^+$ ion and m/z 184 is a fragment ion [19]. The smaller peak **c** (retention time *ca.* 15.7 min) in Figure 2 produced an ammonia CI mass spectrum with m/z 329 as the base peak and minor peaks at m/z 90, m/z 108, m/z 184 and m/z 240. The m/z 329 peak corresponds to the $[MH]^+$ ion for acetylated hydroxydoxylamine (molecular weight 328) and the fragment ions agree well with those reported recently for this compound when analyzed by ammonia CIMS [13]. The smaller GC peaks **b** (15.5 min) and

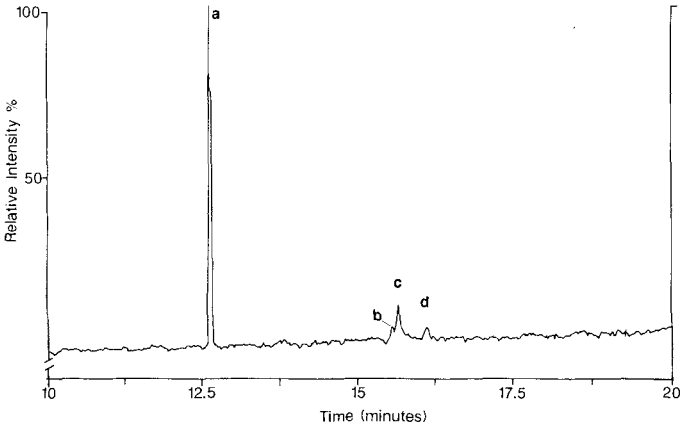

Figure 2

Mass chromatogram of peak 5 in Figure 1B following acetylation. The identifications are:
peak a is doxylamine;
peak b is acetylated didesmethyldoxylamine;
peak c is acetylated hydroxydoxylamine;
peak d is acetylated desmethyldoxylamine.

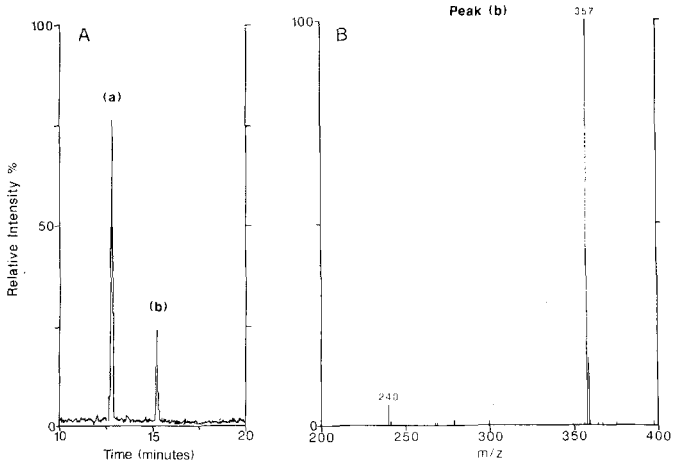

Figure 3

A) Mass chromatogram of peak 6 in Figure 1B following acetylation: GC conditions were oven temperature held at 60 °C for 1 min after injection and then programmed to 300 °C at 20 °/min. The identifications are:
peak a is acetylated desmethyldoxylamine;
peak b is acetylated hydroxydesmethyldoxylamine.
B) The ammonia CI mass spectrum of peak b.

d (16.1 min) shown in Figure 2 produced ammonia CI mass spectra with m/z 285 and m/z 299 as the base peaks, respectively. The m/z 285 peak corresponds to the $[MH]^+$ ion for acetylated didesmethyldoxylamine and the m/z 299 peak corresponds to the $[MH]^+$ ion for acetylated desmethyldoxylamine, and these ammonia CI mass spectra also agree well with those previously reported for these compounds [12, 14]). Although peaks **b** and **d** are minor in the isolated fraction # 5 from the HPLC separation, they were separated and detected by the fused silica GC/MS method. Therefore, the results are consistent with the hypothesis that the second urinary extract fraction (Figure 1A, peak 2) contained doxylamine *O*-glucuronide before incubation with intestinal microflora.

Figure 3A illustrates the GC/mass chromatogram from HPLC fraction # 6 (shown in figure 1B). As indicated in the figure caption this analysis was performed under slightly different GC conditions. The main peak **a**, in this mass chromatogram with a retention time of 12.4 min, exhibited an ammonia CI mass spectrum with a base peak at m/z 182, and a major peak at m/z 299. The m/z 299 peak corresponds to the $[MH]^+$ ion for acetylated desmethyldoxylamine. As shown in **Figure 3B,** a smaller peak **b** inthe mass chromatogram with a retention time of 15.1 min exhibited an ammonia CI mass spectrum with a small peak at m/z 240, and a major peak at m/z 357. The m/z 357 peak corresponds to the $[MH]^+$ ion of acetylated hydroxydesmethyldoxylamine. Therefore, these results are consistent with the hypothesis that the first urinary extract fraction (Figure 1A, peak 1) contained the metabolite desmethyldoxylamine *O*-glucuronide before incubation with intestinal microflora.

In conclusion, these results demonstrate the utility of GC/MS for separating these acetylated aglycones. The GC/MS technique was useful in the identification of two polar deconjugated metabolites which have not previously been reported to be analyzed using this technique. In general, this same approach should be useful for the identification of other aglycones from deconjugated *O*-glucuronide metabolites of similarly structured antihistamines.

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References

[1] Physicians Desk Reference, 36th Edn. p. 1290 Medical Economics, Oradell, New Jersey (1982).

[2] *B. B. Brown* and *H. W. Werner,* J. Lab. Clin. Med. **33** (1948) 325.

[3] *L. B. Holmes,* Teratology **27** (1983) 277.

[4] *S. Shajiro, O. D. Heinonen, V. Siskind, D. W. Kaufmann, R. R. Manson,* and *D. Slone,* Am. J. Obster. Gynecol. **128** (1977) 480.

[5] *R. W. Smithwells* and *S. Sheppard,* Teratology **17,** (1978) 31.

[6] *C. D. Jackson* and *B. N. Blackwell,* Fund. Applied Tox. (1987), submitted.

[7] *C. D. Jackson* and *B. N. Blackwell,* Fund. Applied Tox. (1987), submitted.

[8] *H. C. Thompson, Jr., A. B. Gosnell, C. L. Holder, P. H.Siitonen, K. L. Rowland,* and *J. L. Cmarik,* J. Anal. Toxicol. **10** (1986) 18.

[9] *W. Slikker, Jr., C. L. Holder, J. R. Bailey, J. F. Young,* and *H. C. Thompson, Jr.,* Teratology **27,** (1983) 77A.

[10] *W. Slikker, Jr., C. L. Holder, G. W. Lipe,* and *J. R. Bailey,* Toxicologist **4** (1984) 10.

[11] *W. Slikker, C. L. Holder, G. W. Lipe, W. A. Korfmacher, H. C. Thompson, Jr.,* and *J. R. Bailey,* J. Anal. Toxicol. **10** (1986) 87.

[12] *C. L. Holder, H. C. Thompson, Jr., A. B. Gosnell, P. H. Siitonen, W. A. Korfmacher, C. E. Cerniglia, D. W. Miller, D. A. Casciano,* and *W. Slikker,* Jr., J. Anal. Toxicol. **11** (1987) 113.

[13] *C. L. Holder, P. H. Siitonen, W. Slikker, Jr., C. J. Branscomb, W. A. Korfmacher, C. E. Cerniglia, H. C. Thompson, Jr.,* and *Aubrey B. Gosnell,* J. Anal. Toxicol, submitted (1987).

[14] *C. L. Holder, W. A. Korfmacher, W. Slikker, Jr., H. C. Thompson, Jr.,* and *A. B. Gosnell,* Biomed. Mass Spectrom. **12** (1985) 151.

[15] *W. Gielsdorf* and *K. Schubert,* J. Clin. Biochem. **19** (1981) 485.

[16] *T. D. Doyle* and *J. Levine,* J. Assoc. Off. Anal. Chem. **51** (1) (1968) 191.

[17] *J. C. Drach* and *J. P. Howell,* Biochem. Pharmacol. **17** (1968) 2125

[18] *M. L. Bastos, G. E. Hakanen, R. M. Young, J. R. Monforte,* and *L. Sunshine,* Clin. Chem. **16** (1970) 931.

[19] *W. A. Korfmacher, C. L. Holder, J. P. Freeman, R. K. Mitchum,* and *A. B. Gosnell,* Org. Mass Spectrom. **20** (1985) 435.

[20] *W. A. Korfmacher, C. L. Holder, C. E. Cerniglia, D. W. Miller, E. B. Hansen, Jr., K. L. Lambert, A. B. Gosnell, W. Slikker, Jr., L. G. Rushing,* and *H. C. Thompson, Jr.,* Spectros. Int. J. **4** (1985) 181.

[21] *W. A. Korfmacher, L. D. Betowski, C. L. Holder,* and *R. K. Mitchum,* J. Anal. Toxicol. **11** (1987) 182.

[22] *L. G. Rushing, A. B. Gosnell, C. L. Holder, W. A. Korfmacher* and *W. Slikker, Jr.,* J. High Resol. Chrom. Chrom. Commun. **9** (1986) 435.

[23] *J. O. Lay, Jr., W. A. Korfmacher, D. W. Miller, P. Siitonen,* and *C. L. Holder,* Biomed. Environ. Mass Spectrom. **13** (1986) 627–632.

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