Gas Chromatographic–Mass Spectrometric Differentiation of Atenolol, Metoprolol, Propranolol, and an Interfering Metabolite Product of Metoprolol*

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Abstract

Over a 10-year period, 1993–2002, Federal Aviation Administration identified 50 pilot fatalities involving atenolol, metoprolol, and propranolol, which is consistent with the fact that these drugs have been in the lists of the top 200 drugs prescribed in the U.S. In a few of the 50 pilot fatality cases, initial analysis suggested the presence of atenolol and metoprolol. However, there was no medical history with these cases supporting the use of both drugs. Therefore, atenolol, metoprolol, and/or propranolol, with their possible metabolite(s), were re-extracted from the selected case specimens, derivatized with pentafluoropropionic anhydride (PFPA), and analyzed by gas chromatography–mass spectrometry (GC–MS). The MS spectra of these three antihypertensives and a metoprolol metabolite are nearly identical. All of the PFPA derivatives had baseline GC separation, with the exception of a metoprolol metabolite product, which co-eluted with atenolol. There were four primary mass fragments (m/z 408, 366, 202, and 176) found with all of the PFPA-beta-blockers and with the interfering metabolite product. However, atenolol has three unique fragments (m/z 244, 172, and 132), metoprolol has two unique fragments (m/z 559 and 107), propranolol has four unique fragments (m/z 551, 183, 144, and 127), and the metoprolol metabolite product has two unique fragments (m/z 557 and 149). These distinctive fragments were further validated by using a computer program that predicts logical mass fragments and performing GC–MS of deuterated PFPA-atenolol and PFPA-propranolol and of the PFPA-alpha-hydroxy metabolite of metoprolol. By using the unique mass fragments, none of the pilot fatality cases were found to contain more than one beta-blocker. Therefore, these mass ions can be used for differentiating and simultaneously analyzing these structurally similar beta-blockers in biological samples.

Introduction

Pilots who successfully control their hypertension with medication, diet, and/or exercise can be medically certified to fly aircraft. However, these pilots are considered hypertensive and are closely monitored by the Federal Aviation Administration (FAA) to ensure that their hypertension is properly controlled. Approximately 8% of the active pilots fall under the category of “hypertensive with medication”. During the investigation of fatal civil aviation accidents, postmortem samples obtained from pilots are submitted to the FAA's Civil Aerospace Medical Institute (CAMI) for toxicological evaluation (1). During such evaluation, submitted samples are analyzed for prescription and nonprescription drugs (2), and it is common to find beta-blocker antihypertensives such as atenolol, metoprolol, and propranolol in the submitted biological samples. This observation is consistent with the fact that 28.7% of the U.S. general population have been diagnosed with hypertension (3), and these three antihypertensives have been in the lists of the top 200 drugs dispensed in the U.S. (4,5).

During the 10-year period of 1993–2002, postmortem samples from 3290 civil aviation accident pilot fatalities (cases) were received by CAMI. Toxicological evaluation of these cases revealed that 50 of the 3290 fatalities had the commonly prescribed beta-blockers, atenolol, metoprolol, and propranolol. Out of the 50 fatalities, atenolol, metoprolol, and propranolol was found to be present in 24, 19, and 7 fatalities, respectively, but the initial analysis indicated the presence of atenolol and metoprolol in 4 of these pilot fatalities. Because (i) the combined use of both drugs was not consistent with the history of the drug use by those pilots, (ii) it is uncommon to simultaneously prescribe 2 beta-blockers, and (iii) these commonly used antihypertensives have considerable amount of chemical and structural similarity (6), further examination was undertaken for those fatality cases wherein atenolol and metoprolol were initially detected. Such examination entailed selectively and simultaneously analyzing the three commonly used beta-
were prepared in bovine whole blood from their 1.0 mg/mL.

Materials and Methods

Materials

All chemicals, reagents, and solvents were purchased from commercial sources in the highest possible purity and were used without any further purification. Specifically, the beta-blockers atenolol, metoprolol, and propranolol were purchased from Sigma Chemical Company (St. Louis, MO); N-methyl-4-(4-bromophenyl)-1,2,3,4-tetrahydro-1-naphthylamine (internal standard) was purchased from Pfizer, Inc. (Groton, CT); pentafluoropropionic anhydride (PFPA) was supplied by Pierce, Inc. (Rockford, IL); and atenolol-α and propranolol-α were obtained from Cerilliant Corporation (Round Rock, TX). The alpha-hydroxy metabolite of metoprolol was kindly provided as a gift by AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, U.K.). Bovine whole blood was obtained from Mikkelson Beef, Inc. (Oklahoma City, OK).

All aqueous solutions of drugs, chemicals, and reagents were prepared in double deionized water (DDW) obtained from Milli-Q plus Ultra-Pure Reagent Water System (Millipore, Continental Water Systems, El Paso, TX). A 500-ng/mL solution of the internal standard was also prepared in DDW. Positive controls of the beta-blockers at a concentration of 200 ng/mL were prepared in bovine whole blood from their 1.0 mg/mL methanolic standards. These controls were prepared in pools large enough to provide replicates for the entire study. Bovine blood was also used for negative controls. All samples were stored at −20°C until analyzed.

Sample preparation and extraction procedure

The beta-blocker positive cases identified by the CAMI Toxicology database for reanalysis using our newly developed method spanned a two-year period. In all cases, blood was stored at −20°C in tubes containing 1% (w/v) sodium fluoride/potassium oxalate until analysis. All other specimens, which included urine, liver, kidney, and heart, were stored without preservation at −20°C until analysis.

Tissue specimens were homogenized in DDW (1:2, w/v) by using a PRO250 post-mounted homogenizer (Pro Scientific, Oxford, CT), equipped with a generator (10-mm diameter) set at 22,000 rpm. Three-milliliter aliquots of controls and specimen fluids and 3.0-g aliquots of tissue homogenates were transferred to individual 16 x 150-mm screw top glass test tubes, and 1.0 mL (500 ng/mL) of the internal standard solution was added to each tube. Mixtures in the tubes were vortex mixed briefly and allowed to stand for 10 min. To each of the tubes, except those containing urine, was added 9.0 mL of ice-cold acetonitrile (4°C), and the contents in the tubes were mixed on a rotary mixer for 15 min. Subsequently, the tubes were centrifuged at 820 × g for 5 min to remove cellular particulate matter, including precipitated protein. The supernatants in the tubes were then transferred to 16 x 100-mm culture tubes and evaporated to less than 1 mL in a 40°C water bath under a stream of dry nitrogen. To all the tubes, including the tubes that contained urine samples, was then added 4.0 mL of 0.10M phosphate buffer (pH 6.00).

The buffered mixtures were transferred to solid-phase extraction columns (Bond Elute Certify columns, Varian, Harbor City, CA), which were pre-conditioned with 2.0 mL methanol, followed by 2.0 mL of the 0.10M phosphate buffer. A column flow rate of 1–2 mL/min was maintained in each step by using a Varian 24-port positive pressure manifold (Varian) with approximately 3 psi of nitrogen. Once the extracts had passed through, the columns were washed with 1.0 mL of 1.0M acetic acid, followed by 6.0 mL of methanol. The columns were dried after each wash for 5 min with 25-psi nitrogen. Analytes were eluted off the columns with 4.0 mL of the freshly prepared 2% ammonium hydroxide in ethyl acetate and the eluents were collected in conical glass test tubes.

The eluants collected in the test tubes were then evaporated to dryness in a water bath at 40°C under a stream of dry nitrogen, and subsequently, PFPA (50.0 μL) and ethyl acetate (50.0 μL) were added to each tube. These tubes were then capped, vortex mixed briefly, and incubated in a heating block set to 70°C for 20 min for the PFPA-derivatization. After removing the tubes from the heating block and allowing them to cool to ambient temperature, the reaction mixtures were evaporated to dryness in the 40°C water bath. The residues were then reconstituted in 50.0 μL of ethyl acetate and transferred to autosampler vials for analyses.

Gas chromatographic–mass spectrometric (GC–MS) conditions

Analyses were performed by using a bench-top GC–MS system, consisting of a Hewlett-Packard (HP) 6890 series GC, interfaced with an HP 5973 quadrupole MS (Agilent Technologies, Palo Alto, CA). The GC–MS system was operated with a transfer line temperature of 280°C and a source temperature of 230°C. The GC separation of the analytes was achieved by using a HP-ULTRA-1 crosslinked 100% methyl silicone capillary column (12 m × 0.2-mm i.d., 0.33-μm film thickness, Agilent Technologies). Helium was employed as the carrier gas at a flow rate of 1.0 mL/min. An HP 6890 autosampler was used to inject 1.0 μL of PFPA-derivatized products in ethyl acetate onto the GC–MS system. The GC was equipped with a split/splitless injection port operated at 250°C in the splitless mode with the purge time of 0.5 min. The GC oven temperature profile was 70–290°C at 30°C/min, with a final hold time of 2.67 min, resulting in a total run time of 10 min. Initially, PFPA-derivatized standards of each analyte (1.0 μL of a 100.0 ng/μL ethyl acetate solution) were injected individually and analyzed using the full MS scan mode of 40 to 800 atomic mass units. Subsequently, the MS was operated in selected ion monitoring mode with a dwell time of 30 ms.

Analysis acceptability criteria

Mass ions used for the identification and confirmation of each analyte were selected based on their abundance, mass-to-charge ratio (m/z), and uniqueness (Table I). Acceptability criteria employed for the analysis were (i) ion ratios for a given analyte, measured as the peak area of a qualifier ion divided by the
peak area of the confirmation ion, were required to be within ±20% of the average of the ion ratios for the respective controls analyzed during that analysis; (ii) each ion monitored was required to have a minimum signal-to-noise ratio of 5; and (iii) the analyte was required to have a retention time within ±0.20 min of the average retention time for the respective controls analyzed during that analysis. Any analysis of a particular analyte not meeting the aforementioned criteria was considered as either being negative or inconclusive for the analyte.

Mass fragment prediction

The distinctive fragments chosen for each analyte were validated for their uniqueness by using a computer program that predicted logical mass fragments of chemical compounds (HighChem Mass Frontier™, Thermo Finnigan Corp., San Jose, CA). The predicted mass fragments were also subsequently confirmed by using the mass fragment patterns of the PFPA-derivatized atenolol-$d_5$, propranolol-$d_5$, and alpha-hydroxymetoprolol.

Results and Discussion

The re-examination of the full scan mass spectral data of those fatality cases, wherein atenolol and metoprolol were initially suspected, revealed that the apparent atenolol peak was not atenolol, but it was most likely a co-eluting metabolite product of metoprolol. Such near misidentification highlighted a need for the development of a selective analytical method that can differentiate commonly used, chemically/structurally similar beta-blockers and their possible interfering metabolite(s)/product(s). In the present study, a GC–MS method is described that selectively distinguishes the three beta-blockers from each other and from an interfering metabolite product of metoprolol.

Under the adopted instrumental conditions, the retention times for metoprolol, atenolol, and propranolol were 5.50, 5.74, and 5.80 min, respectively (Table I), suggesting a baseline separation (Figure 1). However, a metoprolol metabolite product interfered with atenolol, as this product co-eluted with the beta-blocker. The mass spectra of these four PFPA-analytes were nearly identical, as there were four dominating common fragments (m/z 408, 366, 202, and 176) with the three beta-blockers and the interfering metabolite product (Figures 2–5). Given the retention times and the four dominating mass fragments, the metoprolol metabolite product could easily be misidentified as atenolol. This limitation was the reason for initially suspecting both atenolol and metoprolol in the pilot fatality cases. However, further in-depth examination of the mass spectral characteristics and chemical fragments of the analytes revealed the presence of three unique fragments (m/z 244, 172, and 132) and the interfering metabolite product.
for atenolol, two unique fragments (559 and 107 m/z) for metoprolol, four unique fragments (m/z 551, 183, 144, and, 127) for propranolol, and two unique fragments (m/z 557 and 149) for the interfering metoprolol metabolite product. The uniqueness of the mass ions was further confirmed by the Thermo Finnigan HighChem Mass Frontier computer program and by the PFPA-atenolol-d7, PFPA-propranolol-d5, and PFPA-alpha-hydroxymetoprolol GC-MS analyses.

The three major urinary metabolites of metoprolol are O-demethylated and oxidized, oxidative-deaminated, and aliphatic hydroxylated products (7,8). The first two metabolites have a carboxyl group and a secondary aliphatic hydroxyl group, but one of these two metabolites also has a secondary amino group. The third metabolite has two aliphatic hydroxyl groups and one secondary amino group. Based on the extraction and GC procedures adopted in this study and the selected mass fragment pattern of the interfering peak, the two acid metabolites were ruled out as possible interfering substances with atenolol. The third hydroxyl and amino group containing metabolite (alpha-hydroxymetoprolol) has three sites that can react with PFPA and, thereby, can generate a product with three CF3CF2CO-moieties. Approximately 0.11 min prior to the atenolol-interfering peak, there was also a non-interfering peak that had a parent ion and a mass fragmentation pattern consistent with that expected for the (CF3CF2CO)3-hydroxyl/amino metoprolol metabolite, that is, alpha-hydroxymetoprolol, but the analyte that co-eluted with atenolol had mass fragments similar to those of a (CF3CF2CO)2-hydroxyl/amino metoprolol product, having a keto group in place of the hydroxyl group of alpha-hydroxymetoprolol. The presence of the (CF3CF2CO)2-metoprolol product could not be explained by the non-derivatization of the alpha-hydroxyl site, since there is no apparent steric hindrance for the site in the molecule. However, it could be possible that the hydroxyl group would have been converted into the keto group because of a chemical rearrangement prior to, or during, the PFPA derivatization and/or the subsequent loss of one of the three CF3CF2CO-groups of the derivatized alpha-hydroxymetoprolol upon exposure to the high temperature at the GC injector port, thus also generating a product with only two CF3CF2CO-groups and a keto group. The mass spectral characteristics of this interfering metabolite product (Figure 5) were further confirmed by analyzing a standard of alpha-hydroxymetoprolol after its PFPA derivatization. During this analysis, the interfering
metabolite product peak was present along with the peak of three PFPA-alpha-hydroxymetoprolol. These findings suggested that the alpha-hydroxy metabolite of metoprolol is partially converted to the interfering, possible keto, product.

Although atenolol was initially identified in a few metoprolol-positive cases, it was not detected in all metoprolol cases. This finding is most likely because the formation of the interfering metabolite product seems to be dependent upon the concentration of alpha-hydroxymetoprolol. In cases where alpha-hydroxymetoprolol was present in small quantities, the interfering product was undetectable and, thus, was not found to contain the atenolol-interfering analyte. Because of this concentration-production relationship, atenolol was initially detected or suspected in only a few metoprolol-positive cases wherein alpha-hydroxymetoprolol was possibly present in large quantities.

Overall, several unique mass fragments reported herein can be used to positively identify the three common beta-blockers and the co-eluting metoprolol metabolite product. By monitoring the selected unique mass fragments and, thereby, taking the selective analytical approach, none of the mentioned pilot fatality cases were found to contain more than one beta-blocker. Therefore, the analytical method reported in this study can be suitably adopted for selectively differentiating and simultaneously analyzing these structurally similar beta-blockers in biological samples.

References


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