Identification of Dimetridazole, Ipronidazole, and Their Alcohol Metabolites in Turkey Tissues by Thermospray Tandem Mass Spectrometry

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Dimetridazole and ipronidazole, two veterinary drugs used to treat turkeys, oxidize in vivo to form alcohol metabolites. The parent drugs as well as their metabolites are usually quantitated by high-performance liquid chromatography (HPLC) with ultraviolet detection. The confirmation of identity procedure reported herein uses HPLC for separation followed by thermospray tandem mass spectrometry (MS/MS) of both the parent and the alcohol metabolites. The extraction and cleanup procedures of the regulatory method were used to isolate the nitroimidazoles from turkey muscle tissue fortified at 2 and 10 ppb. An HPLC thermospray MS/MS technique, operating in the daughter ion mode, was used to confirm the identity of dimetridazole, the alcohol metabolite of dimetridazole, ipronidazole, and its alcohol metabolite. Unfortified control tissues were also analyzed and showed no interference.

INTRODUCTION

The veterinary drugs dimetridazole and ipronidazole were approved for use in turkeys by the U.S. Food and Drug Administration (FDA) in 1971 and 1965, respectively. These two drugs were used in the United States to treat histomoniasis ("black-head") or coccidiosis in turkeys; they were also used as growth promotors. Recently, both drugs were withdrawn from the market in the United States by the manufacturers: dimetridazole in 1987 and ipronidazole in 1989. Canada allows the use of dimetridazole for the treatment of trichomoniasis in turkeys and swine and as a growth promotor in swine. New Zealand, Australia, and Denmark also permit the use of dimetridazole in food-producing animals. Because of the significant importation of meat from these countries, dimetridazole residues could possibly reach the U.S. marketplace. Therefore, suitable methodology needs to be available for monitoring tissues for residues of these nitroimidazoles and their metabolites.

The FDA requires regulatory analytical methods to be both quantitative and capable of confirming the identity of the drug residues. Mass spectrometry (MS) has traditionally been used to clearly define the chemical nature of residues of veterinary drugs approved for use in food-producing animals. In this study, MS was employed to confirm the identity of these compounds.

Dimetridazole (DMZ, 1,2-dimethyl-5-nitroimidazole) has a molecular weight of 141 and an elemental composition of $C_5H_7N_3O_2$. In turkeys, DMZ oxidizes to form the alcohol 2-(hydroxymethyl)-1-methyl-5-nitroimidazole (DMZ-OH), which has a molecular weight of 157 and an elemental composition of $C_5H_7N_3O_3$. Further oxidation forms the acid metabolite, 1-methyl-5-nitroimidazole-2-carboxylic acid. Craine et al. (1974) reported that the primary metabolite found in tissues is the alcohol metabolite, with lesser amounts of the parent compound and the acid metabolite. The official regulatory method (*Code of Federal Regulations*, 1965) for the determination of DMZ is based on polarographic analysis. Both DMZ and DMZ-OH are thermally labile and light sensitive. The photodecomposition as well as other aspects of the chemical nature of these compounds was reported by MacDonald et al. (1971). Craine et al. (1974) developed a polarographic method for determining DMZ-OH in swine at levels as low as 2 ppb. Recently, Carignon et al. (1988) used high-performance liquid chromatography (HPLC) to measure DMZ in pork tissue. HPLC has also been successfully applied to the separation of DMZ from swine feeds by Roybal et al. (1987). Determination of these compounds by gas chromatography (GC) requires highly polar liquid phases. Current methods for quantitation by GC use flame ionization and electron capture detection (ECD).

Ipronidazole (IPN, 2-isopropyl-1-methyl-5-nitroimidazole) has a molecular weight of 169 and an elemental composition of $C_7H_{11}N_3O_2$. In turkeys, IPN is oxidized to the alcohol 1,α,α-trimethyl-5-nitroimidazole-2-methanol (IPN-OH), which has a molecular weight of 185 and an elemental composition of $C_7H_{11}N_3O_3$. These nitroimidazoles are also thermally labile and light sensitive. The FDA regulatory method for the determination of IPN (*Code of Federal Regulations*, 1988) at the 2 ppb level is based on GC separation and ECD. IPN isolated from tissues has been determined by GC by DiSimone et al. (1981). MacDonald et al. (1971) also used GC to determine IPN and IPN-OH isolated from turkey tissue at a level of 2 ppb. Roybal et al. (1987) obtained good recoveries at various drug concentrations by using a quantitative HPLC method to determine IPN isolated from swine feeds.

Various HPLC methods are effective for measuring low levels of DMZ and IPN isolated from either tissues or feeds. Some of these methods could be modified to allow the simultaneous determination of the alcohol metabolite of either DMZ or IPN.

For confirmation of identity by gas chromatography/mass spectrometry (GC/MS), Garland et al. (1980) reported a negative ion chemical ionization procedure that measured IPN and its alcohol metabolite by single-ion monitoring of the molecular anions and their deuterated analogues. This method uses a 100-g tissue extract and can "detect" analytes at the 2 ppm level; however, only one ion is used for confirmation of identity. Morris et al. (1987) quantitated and confirmed the identity of DMZ and IPN isolated from swine feed by GC/MS using multiple-ion monitoring. This method, which uses GC

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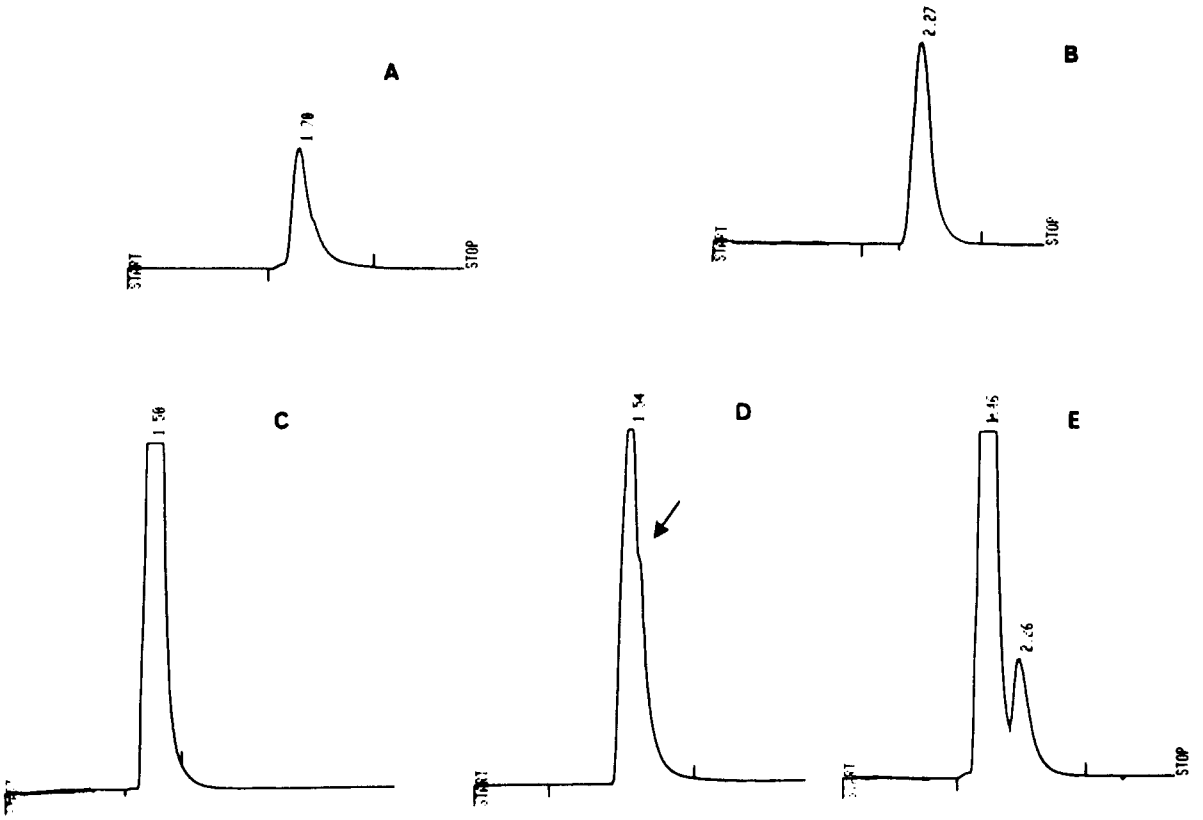

Figure 1. HPLC chromatogram of (A) DMZ-OH, (B) DMZ, (C) control-tissue extract, (D) control tissue fortified with 2 ppb of DMZ-OH, and (E) control tissue fortified with 2 ppb of DMZ. Retention times of chromatographic peaks: (A) 1.70 min; (B) 2.27 min; (C) 1.50 min; (D) 1.54 min; (E) 1.46 and 2.26 min.

separation and electron ionization (EI), monitors five ions for the confirmatory identification of DMZ and six ions for the confirmatory identification of IPN.

The thermospray interface was developed as an introduction system that provides coupling of HPLC to MS. This soft-ionization technique gives molecular ion or molecular adduct ion information and less fragmentation than does the more traditional EI technique. Because many of the newer analytical methods for determining veterinary drug residues use HPLC separations, analyzing the various HPLC eluates by thermospray MS would be advantageous. Using the same extract for both quantitation and confirmation of identity is a more credible and efficient approach than using different extraction procedures for different instrumental techniques to quantitate and confirm the identity of the drug of interest. Because thermospray MS normally produces only molecular ion (or adduct ion) information, the technique lacks specificity; that is, there is insufficient fragmentation to characterize the compound of interest. One way to produce sufficient fragmentation to fully characterize a compound is to use tandem mass spectrometry (MS/MS).

In this paper, the results of HPLC separation thermospray MS/MS are presented for DMZ, IPN, and their alcohol metabolites isolated from turkey tissues. Control and fortified-control turkey muscle tissues were extracted by either the regulatory method or the drug sponsor's method. The tissues were fortified at 2 and 10 ppb. The presence of drug in the tissue extracts was verified by HPLC with ultraviolet (UV) detection before thermospray MS/MS analysis.

EXPERIMENTAL PROCEDURES

Reagents. The qualities of the reagents used in the tissue extraction and cleanup were those specified in each method; solvents were of at least pesticide residue quality. The standards, DMZ, DMZ-OH, IPN, and IPN-OH, were used as received from the manufacturer.

Tissue Extraction and Cleanup. *DMZ and Its Metabolite.* Control and fortified-control turkey breast tissues were prepared according to a modification of the method of Craine et al. (1974). The final acid extract was made basic with 3 g of potassium monohydrogen phosphate, and the analytes were extracted into methylene chloride. The organic solvent was evaporated and the residue redissolved in the HPLC mobile phase.

IPN and Its Alcohol Metabolite. Control and fortified-control turkey muscle tissues were prepared according to the official method (*Code of Federal Regulations*, 1988). The compound was extracted from the tissues with benzene in the presence of borax. The extract was purified by using a silica column. The final benzene extract was evaporated to dryness and then redissolved in HPLC mobile phase.

Instrumentation. A Finnigan thermospray (San Jose, CA) was interfaced to a Finnigan Model TSQ 46 triple-stage quadrupole mass spectrometer. Before introduction into the thermospray source, the drugs or metabolites were separated by HPLC using a Spectroflow 400 pump (Kratos, Ramsey, NJ), a Rheodyne Model 7125 injection valve (Cotati, CA), and a Whatman Partisil 5 C_8 RAC II 10-cm column (Clifton, NJ). The mobile phase consisted of water/methanol (50:50) containing 0.1 M ammonium acetate; the flow rate was 1.0 mL/min. In the mass spectrometer, ionization was by thermospray using the ammonium acetate buffer. The following operating conditions were used: vaporizer tip, 120 °C; jet temperature (source block), 230 °C; preamplifier, 10^{-8} A/V; conversion dynode, 5 keV; electron multiplier, 1400 eV. For these experiments, the three sets of quadrupole rods (Q1, Q2, Q3) were configured to perform daughter ion analysis. Q1 was set to transmit ions of a particular m/z (protonated molecular ion) into Q2, which served as a collision cell. The ions selected in Q1 collided with the argon reagent gas to produce collisionally activated decomposition fragment ions, which were scanned in Q3. For the MS/MS daughter ion experiments, the collision energy was set at −12 eV and the pressure for the argon collision gas was set at 2 mTorr. The third set of quadrupole rods (Q3) scanned from 40 to 200 amu.

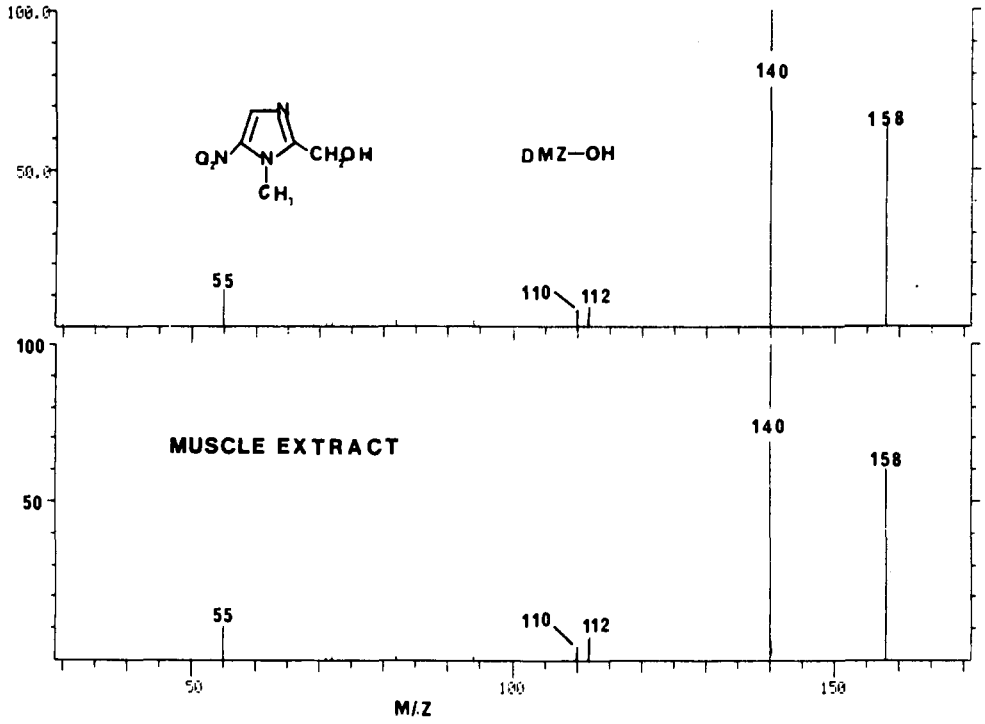

Figure 2. Dual spectra of DMZ-OH (top) and muscle tissue extract fortified with 2 ppb of DMZ-OH (bottom).

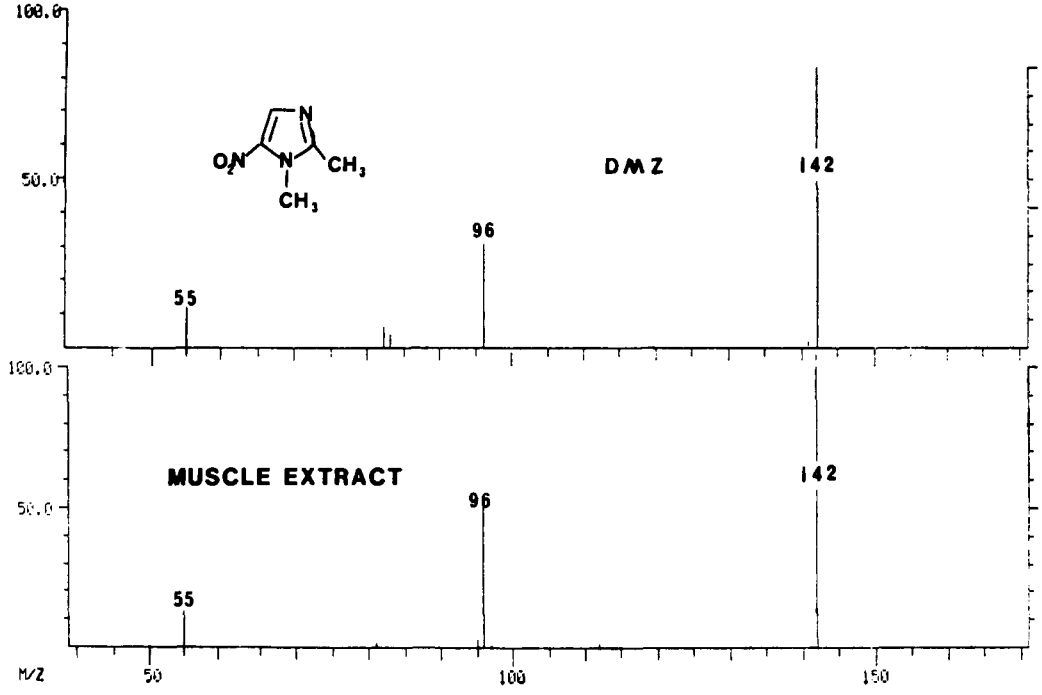

Figure 3. Dual spectra of DMZ (top) and muscle tissue extract fortified with 2 ppb of DMZ (bottom).

For HPLC-UV analysis of the tissue extracts, a Spectroflow 400 pump, a Whatman C_8 column with the mobile phase listed above, and a Spectroflow Model 783 variable-wavelength detector set to monitor at 254 nm and 2 AUFS were used. Under these conditions, the standards had the following retention times: DMZ-OH, 1.70 min; DMZ, 2.27 min; IPN-OH, 1.89 min; IPN, 2.50 min.

Turkey breast muscle was obtained locally. Control muscle and muscle fortified at levels of 2 and 10 ppb were studied. The drugs and their alcohol metabolites were isolated according to the methods presented previously. An aliquot of each of the turkey muscle tissue extracts was chromatographed by reversed-phase HPLC using a UV detector set at 254 nm to verify that the compounds of interest at the 2 ppb level had been isolated. The extract (2 ppb level) was then analyzed by HPLC thermospray MS/MS to confirm the identity of these veterinary compounds.

RESULTS AND DISCUSSION

The HPLC chromatograms presented in Figure 1 are standards of DMZ-OH (A) and DMZ (B), extracts of control tissue (C), control tissue fortified with DMZ-OH (D), and control tissue fortified with DMZ (E). The chromatogram of the control-tissue extract (C) shows a peak for a component that eluted at about 1.50 min, which could interfere with the HPLC-UV determination of DMZ. However, in the MS/MS analysis, the selectivity of Q1 eliminated this possible interference because the tandem mass spectrometer gave no response at m/z 158.

The MS/MS daughter ion spectra of DMZ-OH and DMZ show similar fragmentation patterns. Both show losses of NO_2 from their protonated molecular ion and ring cleavage

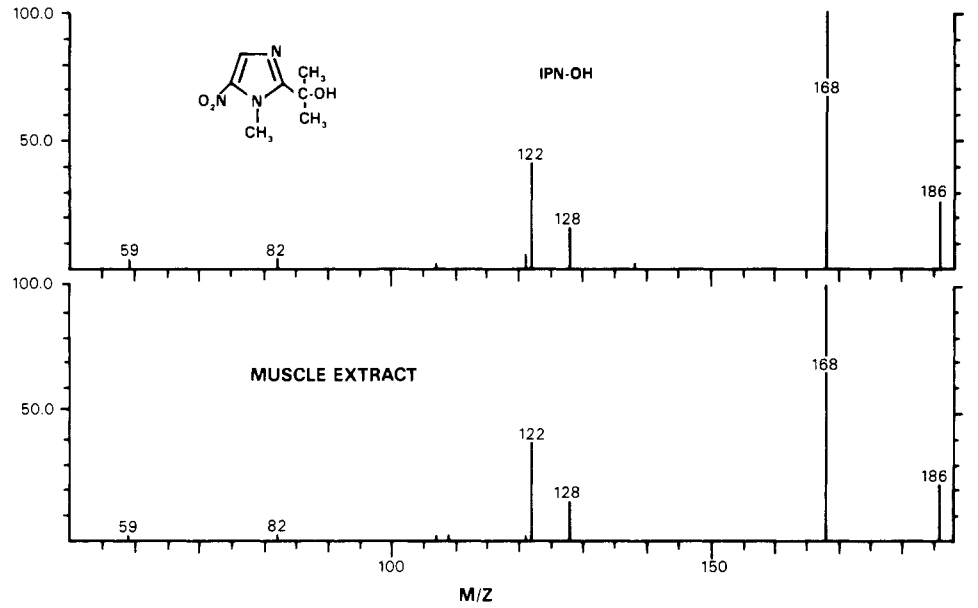

Figure 4. Dual spectra of IPN-OH (top) and muscle tissue extract fortified with 2 ppb of IPN-OH (bottom).

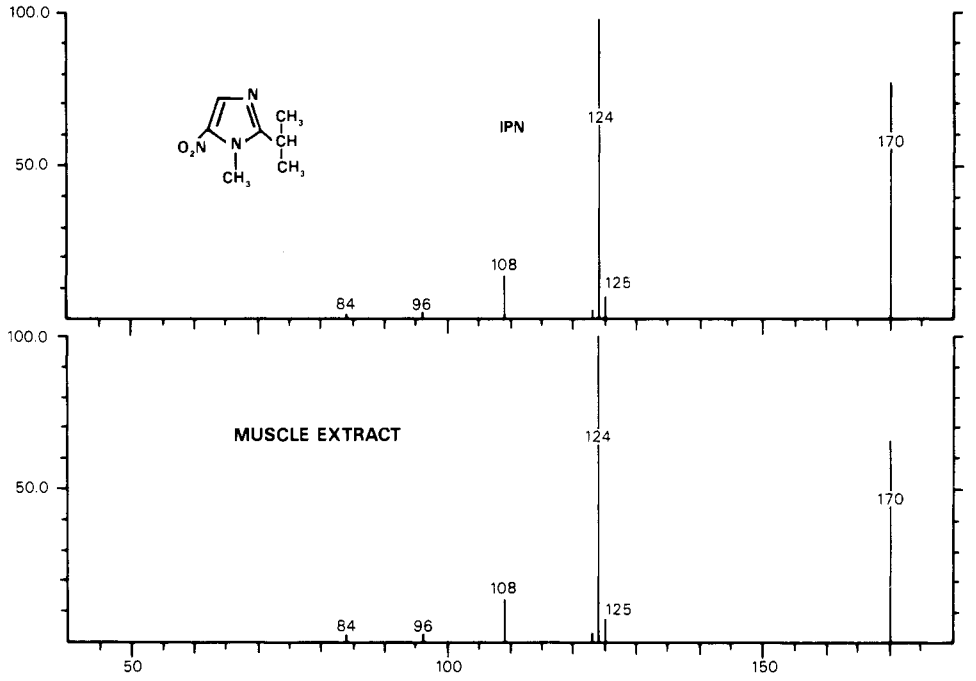

Figure 5. Dual spectra of IPN (top) and muscle tissue extract fortified with 2 ppb of IPN (bottom).

to yield an ion consistent with the formula C_3H_5N. The DMZ-OH spectrum demonstrates additional losses of H_2O and hydrogen. DMZ-OH elutes at 1.7 min. The MS/MS spectra of the DMZ-OH standard and the fortified-tissue extract are shown in Figure 2. The protonated molecular ion is present at m/z 158. The base peak is the ion at m/z 140 and is postulated to result from the loss of H_2O from the alcohol functionality. The loss of NO_2 or 46 Da from the protonated molecular ion forms the ion at m/z 112. The loss of two hydrogen atoms from the ion at m/z 112 accounts for the ion at m/z 110. The ion at m/z 55 emanates from a ring cleavage of the nitroimidazole to yield a fragment ion C_3H_5N. DMZ eluted from the HPLC column after its alcohol metabolite at approximately 2.27 min. The spectra of DMZ and the fortified-tissue extract are presented in Figure 3. The protonated molecular ion and base peak at m/z 142 lose NO_2 to form the ion at m/z 96. As postulated for the metabolite spectra, the ion observed at m/z 55 (C_3H_5N) formed from the imidazole ring cleavage.

The fragmentation patterns of IPN-OH and IPN are similar in that NO_2 is lost. Additional daughter ion studies were performed on the fragment ions of IPN-OH to investigate the pathways. IPN-OH eluted from the HPLC column at 1.89 min. Figure 4 illustrates spectra of the standard and the muscle extract. In these spectra, the protonated molecular ion at m/z 186 loses water to form an ion at m/z 168. The loss of NO_2 accounts for the ion at m/z 122. The ion at m/z 128 could result from the loss of the 2-substituted 2-propanol (C_3H_6O) from the molecular ion. The ion at m/z 82 may be formed by the loss of NO_2 from the ion at m/z 128. The loss of 2-propanol from the nitroimidazole forms the ion at m/z 59. This proposed fragmentation pattern was verified by additional daughter ion experiments performed at 168, 128, and 122 Da on the daughter ions from the IPN standard. The spectra of the daughter ions of m/z 168 showed ions at m/z 122 and 59 indicative of the proposed losses. The spectra of the daughters of the ion at m/z 128 showed the formation of

an ion at m/z 82, representing the loss of 46 Da. This finding further supports the proposed fragmentation scheme. IPN eluted from the C_8 column at approximately 2.50 min. The daughter ion spectra of IPN standard and fortified-muscle extract are shown in Figure 5. The spectrum of ipronidazole exhibits a protonated molecular ion, and the loss of 46 Da forms the base peak m/z 124. The subsequent loss of 16 Da from the ion at m/z 124 forms the ion at m/z 108. The loss of C_2H_4 from the ion at m/z 124 accounts for the ion at m/z 96. The ion at m/z 84 forms from the ring cleavage between carbon atoms 2 and 5 and includes the isopropyl side chain.

The EI spectra of DMZ and IPN have been reported by Morris et al. (1987). Our thermospray findings are consistent with the EI data. The negative ion chemical ionization spectra of IPN and its alcohol metabolite have been published by Garland et al. (1980). The data demonstrate molecular anions only. Thermospray usually produces molecular ions or adduct ions and little fragmentation. The use of the thermospray interface and the ability to perform MS/MS daughter ion analysis of the protonated molecular ion allows a more complete characterization of the structure of the compound than that provided by thermospray alone.

The thermospray tandem mass spectra of the four nitroimidazoles presented here indicate one approach for the characterization of these veterinary drugs and metabolites in muscle tissue extracts. Because many veterinary drugs and their metabolites are polar, HPLC is the preferred analytical technique. The same extract used for the HPLC determinative procedure can also be used for thermospray MS/MS confirmation of identity analysis. These drugs can be identified in tissue extracts fortified at 2 and 10 ppb. The control-tissue extracts did not show any signal at the particular ion being monitored. The increased selectivity of the tandem mass spectrometer allows for exclusion of otherwise potentially interfering peaks. The MS/MS daughter ion analysis defines the structure of the nitroimidazoles.

CONCLUSION

HPLC is the preferred technique for many polar compounds, such as these veterinary drugs and their metabolites. Ideally, the same tissue extract should be used for confirmation of identity studies. In these cases, thermospray is a suitable choice of interface for MS analysis by reversed-phase HPLC separations. Although thermospray MS usually forms only molecular ion or adduct ions, the use of MS/MS adds both selectivity, from its ability to filter out other ions, and definition, from the daughter ion spectra obtained.

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