Determination of Ipronidazole and Its Principal Metabolite in Turkey Skin and Muscle by Combined Gas Chromatography–Negative Chemical Ionization Mass Spectrometry–Stable Isotope Dilution

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A GC–MS procedure is described which can detect ipronidazole and its metabolite, α,α-dimethyl-1-methyl-5-nitro-1*H*-imidazole-2-methanol, in turkey skin and muscle at the 2-ppb level. One hundred grams of turkey tissue is processed using the isolation procedure previously developed for the compound's EC–GC regulatory assay. Portions of the resulting benzene solutions are then analyzed by GC–MS using selective ion monitoring. Methane is used both as GC carrier gas and negative chemical ionization reagent gas. For the determination of ipronidazole and its metabolite, molecular anions at *m/e* 169 and 185, respectively, are monitored in the GC effluent. Deuterated analogues of ipronidazole and its metabolite are added at the 2-ppb level to the tissue before processing to establish the retention times, extraction efficiencies, mass spectral responses, and chromatographic peak shapes of both ipronidazole and its metabolite.

Ipronidazole [2-(1-methylethyl)-1-methyl-5-nitro-1*H*-imidazole, IP, Table I] is the active compound in Ipropan, a medicated premix added to turkey feed for the control of histomoniasis (Mitrovic and Schildknecht, 1970; Marusich et al., 1970). The drug's principal metabolite in turkeys (Fellig et al., 1969) is α,α-dimethyl-1-methyl-5-nitro-1*H*-imidazole-2-methanol (HIP, Table I).

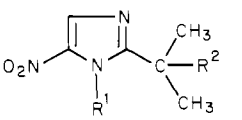
A sensitive and specific EC–GC procedure for IP and HIP in turkey skin and muscle residues, at the regulatory 2-ppb level, has been published (MacDonald et al., 1971). In this paper, we describe a GC–MS method for IP and HIP which is suitable as a confirmatory assay for the EC–GC procedure.

The determination of IP or HIP in tissues at the regulatory level offers several interesting analytical challenges. Exact estimates of compound recoveries are difficult to obtain since both compounds are light sensitive (MacDonald et al., 1971) and will easily undergo hydrolysis under alkaline conditions (Lau et al., 1969). Because of their high vapor pressure, both compounds will evaporate if solutions containing them are extensively concentrated. In addition, the detector used in the assay must possess the great sensitivity of the EC detector without sacrificing that detector's specificity. Specificity is important since both IP and HIP are structurally similar to many compounds present in a biological matrix. To best meet these challenges, we reasoned that a confirmatory assay for IP and HIP should combine the established EC–GC sample workup procedure with stable isotope methodology and negative chemical ionization–mass spectrometry (NCIMS).

Stable isotope analogues of IP and HIP would serve as almost ideal standards for the assay. Addition of these compounds to the tissue before processing would clearly establish the recoveries and retention times of IP and HIP. In addition, a comparison of the peak widths at half-height of the stable isotope analogue and the protio compound offers a means for detecting the presence of interfering, unresolved substances.

The use of NCIMS is attractive for several reasons. Hunt et al. (1976) reported the technique to be extremely sensitive for certain compounds. On the basis of their fine EC responses, both IP and HIP would be expected to show excellent sensitivity with NCIMS. Because most materials in a biological matrix are not ionized by thermal electrons, methane NCIMS provides increased specificity relative to other ionization modes (Dougherty and Hett, 1978).

Table I. Structures of Ipronidazole (IP) and Ipronidazole's Metabolite, HIP, and Their Stable Isotope Analogues, IP-d_3 and HIP-d_3



compd	R1	R2	MW
IP	CH_3	H	169
HIP	CH_3	OH	185
IP-d_3	CD_3	H	172
HIP-d_3	CD_3	OH	188

EXPERIMENTAL SECTION

A. Apparatus. A Finnigan Model 3200 quadrupole mass filter system and Finnigan Promim peak monitor were used in conjunction with the same manufacturer's Model 9500 gas chromatograph.

The glass GC column, 5 ft × 2 mm i.d., was packed with 3% Silar 10C on Gas-Chrom Q (100–200 mesh) obtained from Applied Science Laboratories. Prior to use, the column was conditioned overnight at 250 °C with a 20 mL/min nitrogen flow. For the assay, methane was used both as carrier gas (10 lb/in^2) and CI reagent gas (ion source pressure 1 torr). The injector, column oven, separator oven, and source re-entrant tube were operated at 300, 210, 250, and 225 °C, respectively. Under these conditions, the retention times of IP and HIP were 1.25 and 3.25 min, respectively.

The mass spectrometer was modified to detect negative ions using the method of Stafford et al. (1978). The voltage to the conversion dynode was provided by a Hewlett Packard Model 6516A, 0–3000 V, power supply. Typically, the conversion dynode was operated at +1800 V and the continuous dynode electron multiplier was operated at −2000 V. The ion energy was supplied by a ±30-V power supply (Model 30-.6, Hyperion, Watertown, MA). The electron trap and ion repeller were also connected to this power supply. The lens voltage was provided by a Hewlett

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Packard Model 6209B, ±320 V, power supply. A 300-G magnet was placed outside the vacuum on the quadrupole housing above the ion source and was moved until the small background signal at m/e 35 (^{35}Cl) was maximized.

The ion energy and lens voltages were set to give the maximum signal intensity consistent with optimum peak shape and unit mass resolution. The filament emission and electron energy were set to their lowest values consistent with maximum negative ion production.

Each Promim channel was operated with a 100-ms dwell time, a 0.5-Hz frequency response, and a gain of 10^{+8} V/A. Ion chromatograms were recorded on a four-pen Rikadenki recorder using a paper speed of 2 cm/min.

B. Materials. Most materials have been described by MacDonald et al. (1971). *N*-Trideuteriomethyl analogues of IP and HIP, IP-d_3, and HIP-d_3, were synthesized using the method of Hoffer (1972). *N*-Desmethyl-IP and *N*-desmethyl-HIP were obtained from Dr. W. Scott, Hoffmann-La Roche Inc., Nutley, NJ.

1. 2-(1-Methylethyl)-1-(methyl-$^2H_3$)-5-nitro-1H-imidazole, IP-d_3. *N*-Desmethyl-IP (0.998 mg) and dimethyl-$^2H_6$-sulfate (0.9 mL, >99% ^2H, Aldrich Chemical Co.) were heated at 60 °C for 6 h. Ice water (1.9 mL) was added, followed by methylene chloride (9.55 mL) and cold 3 N NaOH (3.8 mL). Following agitation, the methylene chloride layer was removed, washed twice with cold 3 N NaOH (1.9 mL each time), once with cold water, and dried over magnesium sulfate. The residue after removal of the methylene chloride was crystallized from diethyl ether/acetone to yield in two crops a total of 161.2 mg of the titled compound. The residue from the mother liquor was crystallized from petroleum ether and gave an additional 329.8 mg of the titled compound. IP-d_3 melted at 59–59.5 °C and showed the expected MS and chromatographic properties. The ^1H NMR spectrum was identical with that of the protio compound except for the absence of the *N*-methyl absorption at δ 3.95.

2. α,α-Dimethyl-1-(methyl-$^2H_3$)-5-nitro-1H-imidazole-2-methanol, HIP-d_3. The above procedure was carried out on 488.5 mg of *N*-desmethyl-*HIP*. The residue from the methylene chloride layer was crystallized from petroleum ether/acetone to yield 85.4 mg of the titled compound. An additional 109.4 mg of HIP-d_3 was obtained by crystallization from benzene of the residue from the mother liquor. The compound had a melting point of 98–100 °C and showed the expected MS and chromatographic properties. The ^1H NMR spectrum was identical with that of the protio compound except for the lack of the *N*-methyl absorption at δ 4.19.

C. Solutions. Standard solutions of IP-d_3 and HIP-d_3 were prepared in identical fashion to the IP and HIP standards described by MacDonald et al. (1971). Five additional solutions were prepared in benzene to check the performance of the GC–MS. Solutions A, B, C, D, and E contained 100 ng/mL of both IP-d_3 and HIP-d_3 and 0, 50, 100, 200, and 400 ng/mL of both IP and HIP. These solutions were stored in amber glassware.

D. Mass Spectra. For the determination of IP and IP-d_3, the Promim was set to monitor each compound's molecular anion at m/e 169 and 172, respectively. For the determination of HIP and HIP-d_3, the Promim was set to monitor each compound's molecular anion at m/e 185 and 188, respectively.

E. Procedure. Duplicates of unknown turkey skin and muscle samples (100 g) were analyzed along with duplicates of five control tissue samples each spiked with 0, 100, 200, 400, and 800 ng of both IP and HIP. Prior to analysis, all samples were spiked with 200 ng each of IP-d_3 and HIP-d_3. The spiking volumes and spiking procedures have been described by MacDonald et al. (1971). The samples were extracted and the compound isolated using the method of MacDonald et al. (1971). This procedure produced two 1-mL benzene extracts—one suitable for the analysis of IP and one suitable for the analysis of HIP. The MS was tuned to analyze for IP and IP-d_3, and the extracts suitable for IP analysis were injected.

First, the extracts from the spiked tissue were injected and then the extracts from the unknown samples were injected. The instrument was then tuned to analyze for HIP and HIP-d_3, and the extracts suitable for HIP analysis were injected. For both the IP and HIP analyses, approximately 5 μL out of the available 1000 μL final solution was injected. Thirty seconds after injection, the GC divert valve was turned off, and the electron filament supplies turned on.

The mass spectral tuning procedures for IP or HIP were similar. Several 10-μL injections of solution E were made to tune the mass spectrometer, using the Promim, to monitor the molecular anions of either IP or HIP. The instrument mass marker was then used to set the mass spectrometer to monitor the molecular anions of IP-d_3 or HIP-d_3. With similar injections, the optimum ion source voltage, quadrupole rod polarity, and magnet positions were found, and the column oven temperature was adjusted to give the compounds their desired retention times. Following the tuning procedure, the injection of 10 μL of neat benzene should give no response at the ions of interest.

Aliquots of solutions A, B, C, D, and E were then injected. A 2-μL injection of solution C (200 pg) should give a peak with a S/N >200 for IP and >50 for HIP. Injection of 5 μL of solution A should give a response at the mass corresponding to IP or HIP that is <2% of the response of the IP-d_3 or HIP-d_3 ions. Before injecting the extracts from samples, additional 10-μL injections of neat benzene were made to verify a lack of response at the ions of interest.

Following collection of the data, the heights and widths at half-heights of the ions were measured with a ruler, and the peak height ratios of m/e 169 to m/e 172 and m/e 185 to m/e 188 were determined. The distances between the tops of the peaks corresponding to the protio and deuterio compounds were measured. These distances should be within 20% of the pen offset distance. With the Rikadenki recorder, the pen offset distance typically represents a retention time of 7 s. For each peak in the same sample, the peak width at half-height of the protio compound should be within 3% of the peak width at half-height of the deuterio compound.

The data from the samples spiked with known amounts of IP and HIP were subjected to a linear least squares fitting of ppb (x) vs. ion ratio (y) using an appropriate program on a digital computer. The fit should show a high (>0.98) correlation coefficient. The slope and intercept generated by this procedure should be similar to the slope and intercept generated by an identical least-square fitting of the data from solutions A, B, C, D, and E. The slope (m) and the intercept (b) of the regression line and their standard deviations, SD_m and SD_b, respectively, from the fitting were recorded and used in the equation below to predict an ion ratio (R_L) which, if exceeded, confirmed the presence of IP or HIP in a concentration above the legal limit:

$$R_L = (m - 2SD_m)2 + (b - 2SD_b)$$

If duplicates from unknown samples gave differing results, the assay was repeated.

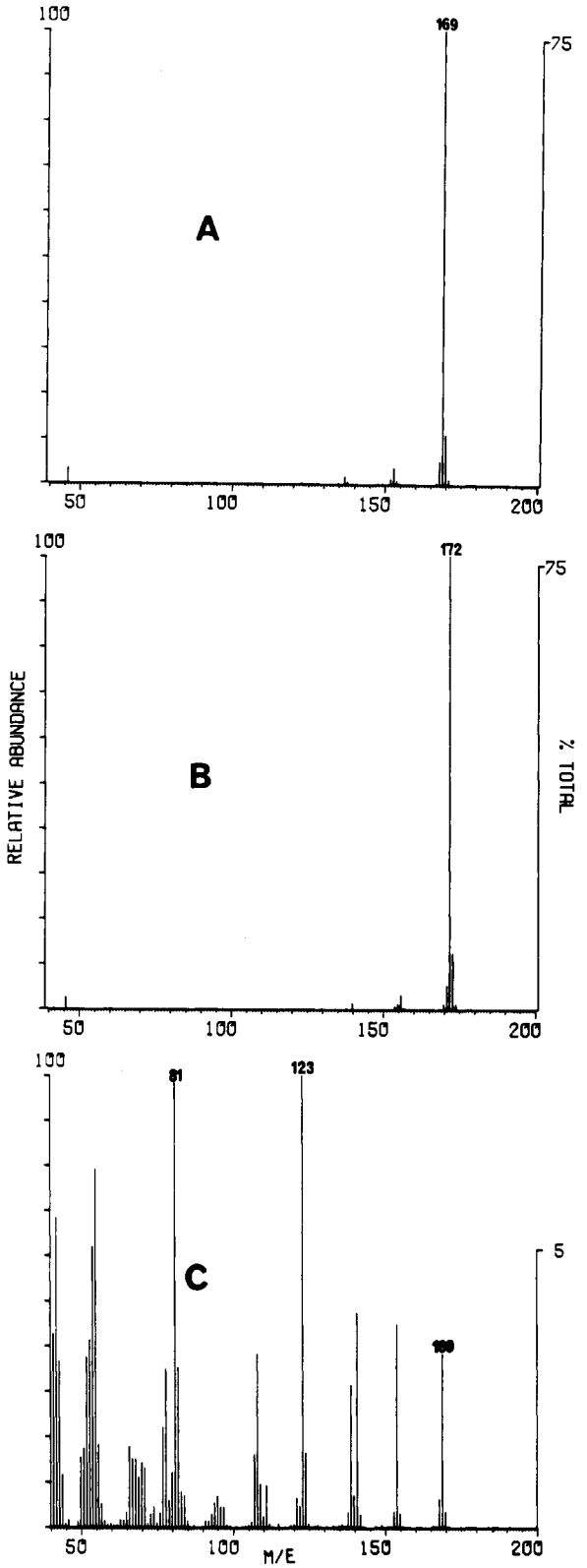

Figure 1. NCI mass spectrum of IP (A) and IP-d_3 (B). Positive ion EI mass spectrum (55 eV) of IP (C).

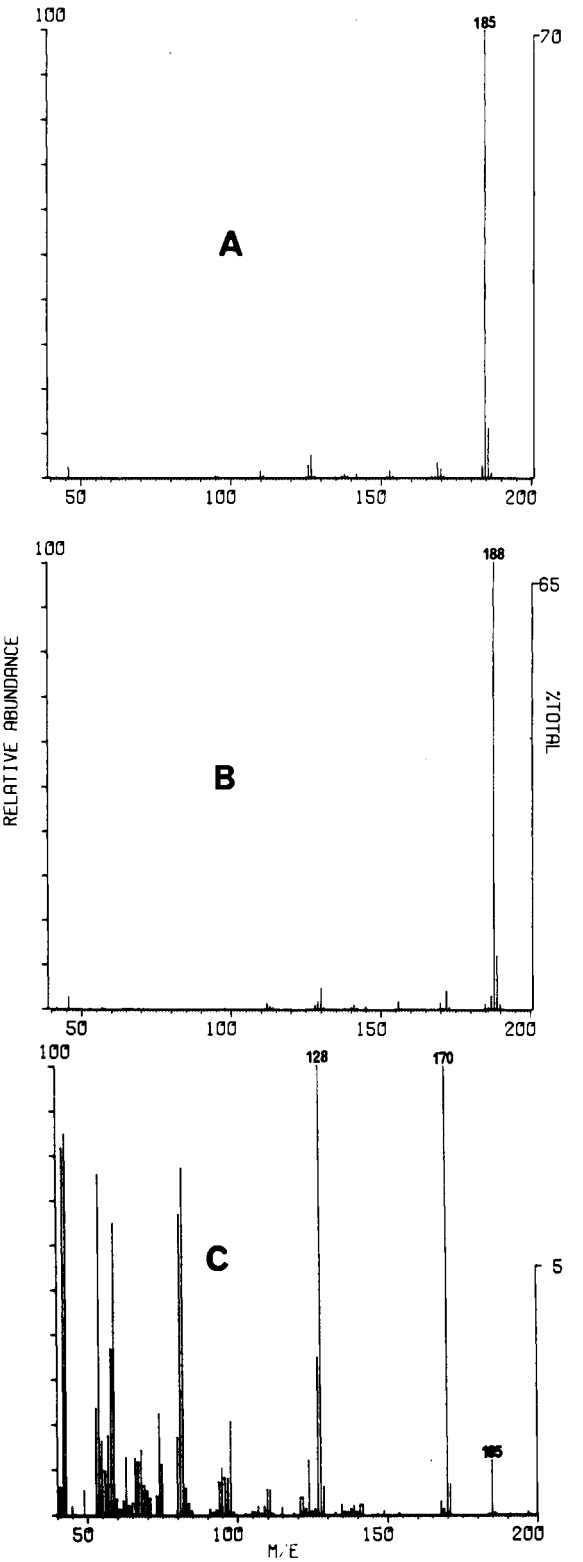

Figure 2. (A) NCI mass spectrum of HIP (A) and HIP-d_3 (B). Positive ion EI mass spectrum (55 eV) of HIP (C).

RESULTS AND DISCUSSION

The principal ion in the NCI spectra of IP, HIP, and their stable isotope analogues was each compound's $M^-\cdot$ molecular anion (Figures 1 and 2). The NCI spectra were desirable in that most of the ion current was carried by an ion expressing the compound's molecular weight, but undesirable in that specificity was limited because only one ion could be used for identification. In the EI mass spectra of these same compounds (Figures 1 and 2), several fragment ions in addition to the $M^+\cdot$ molecular ion were available for compound characterization. However, although it could not be determined exactly because of the separator and "open" source used in GC–EIMS, we estimated that GC–NCIMS was 20 times more sensitive than GC–EIMS for the analysis of IP or HIP. Thus, to utilize the increased sensitivity of NCI, identification had to be based on the occurrence of a signal at a single ion mass at the correct retention time. Accepting this limitation

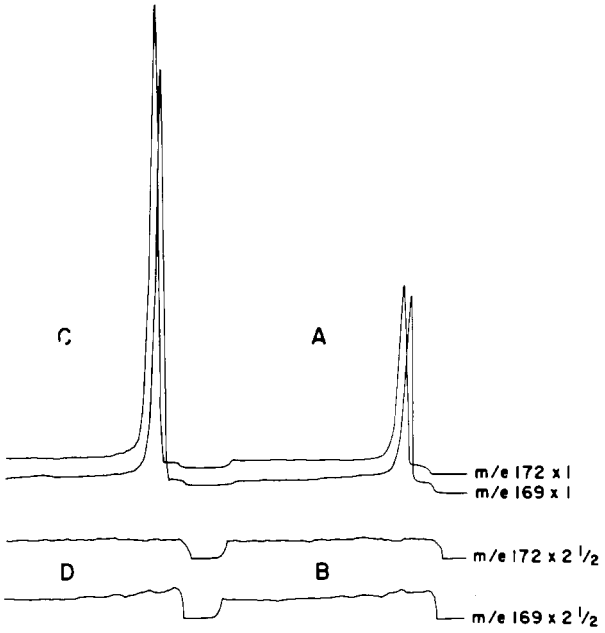

Figure 3. Ion chromatograms from the analyses of control turkey skin spiked with 2 ppb (A) and 0 ppb (B) of both IP (m/e 169) and IP-d_3 (m/e 172) and control turkey muscle spiked with 2 ppb (C) and 0 ppb (D) of both IP and IP-d_3. For ion chromatograms B and D, 5 μL out of the available 1000 μL was injected. For ion chromatograms A and C, 4 and 8 μL, respectively, were injected.

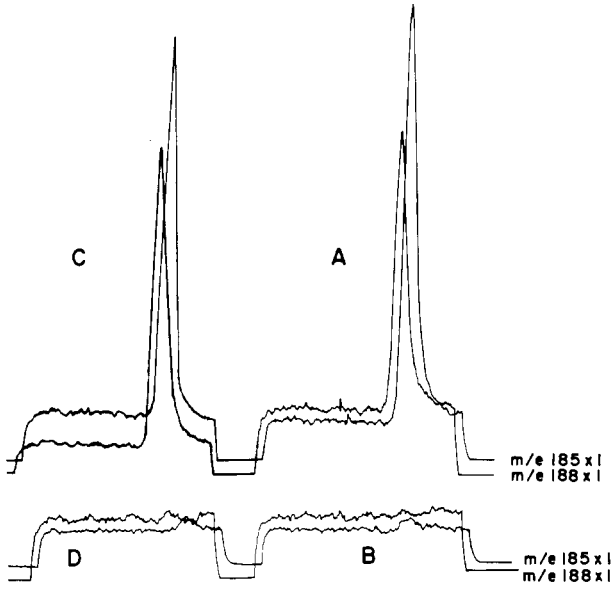

Figure 4. Ion chromatograms from the analyses of both control turkey skin spiked with 2 ppb (A) and 0 ppb (B) of both HIP (m/e 185) and HIP-d_3 (m/e 188) and control turkey muscle spiked with 2 ppb (C) and 0 ppb (D) of both HIP and HIP-d_3. For ion chromatograms B and D, 5 μL out of the available 1000 μL was injected. For ion chromatograms A and C, 2 μL was injected.

placed great importance on the exact determination of the retention time of IP or HIP. However, from the analysis of many samples over several months, we observed that IP and IP-d_3 and HIP and HIP-d_3 typically eluted within 0.2 and 0.8 s, respectively, of one another. Thus, the retention times of IP and HIP could be clearly established by observing the virtually simultaneous, i.e., within 1 s, appearance of the protio and deuterio ions.

As an additional check on the presence of IP or HIP, the peak widths at half-height of the protio and deuterio compounds from the same samples were compared. The peak widths would be different if the response of either ion is complicated by the response from an interfering substance. In the course of analyzing many samples over a period of several months, we found no samples where the peak widths between the protio and deuterio compounds differed by more than 3%.

Typical ion chromatograms from the analyses of turkey skin and muscle spiked with known amounts of IP and HIP are shown in Figures 3 and 4. Two features of these chromatograms were of particular significance. The first was the absence of interfering ions in any of the chromatograms. This fulfilled our hope that most substances in the biological matrix would be insensitive toward methane NCI. The second feature to note was the sensitivity. On the basis of a comparison of the response of the IP-d_3 and HIP-d_3 ions from the injection of solutions B, C, and D and the response of these ions in the extracted samples, we estimated the recoveries to be 95% for IP and 70% for HIP. Using these recoveries, the amounts of IP and HIP injected in the 2-ppb skin samples were approximately 0.4 and 0.2 ng, respectively. The observed IP peak had a signal-to-noise ratio greater than 250:1. The observed peak for HIP had a signal-to-noise ratio greater than 50:1.

Typical standard curves from the assay are shown in Figures 5 and 6. As can be seen, a linear relationship (correlation coefficient >0.99) existed between the amount of IP and HIP added and the observed m/e 169 to m/e 172 and m/e 185 to m/e 188 ion ratios. The assay used

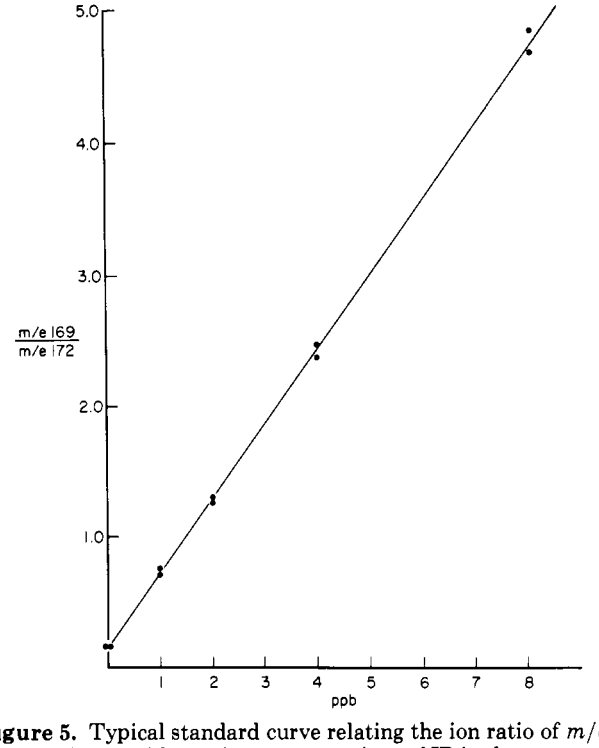

Figure 5. Typical standard curve relating the ion ratio of m/e 169 to m/e 172 with varying concentrations of IP in the presence of a constant 2 ppb of IP-d_3. The line is from a "least squares" fit of the ion ratio versus ppb added data. The slope of the line (±SD) was calculated to be 0.57 ± 0.006 with an ion ratio intercept (±SD) of 0.16 ± 0.02. The correlation coefficient for the linear fit was 0.99.

the slopes and the intercepts of such standard curves and the standard deviations of the slopes and the intercepts to predict, at the 95% confidence level (two SD's), a value for the ion ratio, which if exceeded, confirmed that an unknown sample was in violation of the 2-ppb legal residue limit.

The assay was used to measure IP and HIP in the skin and muscle of a number of turkeys slaughtered following

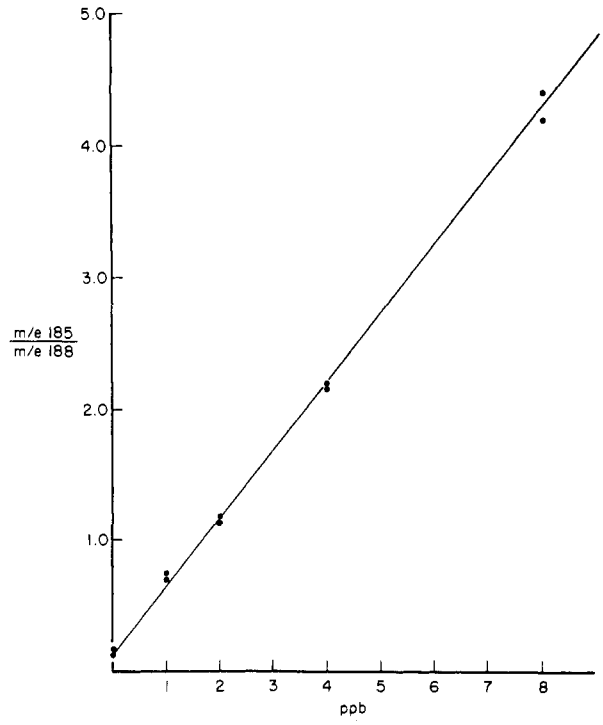

Figure 6. Typical standard curve relating the ion ratio of m/e 185 to m/e 188 with varying concentrations of HIP in the presence of a constant 2 ppb of HIP-d_3. The line is from a "least squares" fit of the ion ratio vs. ppb added data. The slope of the line (±SD) was calculated to be 0.51 ± 0.007 with an ion ratio intercept (±SD) of 0.16 ± 0.03. The correlation coefficient for the linear fit was 0.99.

continuous feeding with Ipropran. Ion chromatograms from a typical tissue assay can be seen in Figure 7. For these assays, the IP-d_3 and HIP-d_3 internal standards were found to have virtually the same peak widths and to elute at virtually the same time as the peaks assigned to IP and HIP, respectively.

For both the IP-d_3 and HIP-d_3 ions, the peaks in the ion chromatogram represented at most 0.12 ng injected. The regulatory EC–GC procedure had suggested the sample contained virtually no IP and somewhat more than 2 ppb HIP. The m/e 169 to m/e 172 ion ratio from the IP and IP-d_3 ion chromatograms was 0.42. The IP standard curve that day had a slope (±SD) of 0.57 ± 0.006 and an ion ratio intercept (±SD) of 0.16 ± 0.02 (Figure 5). Using the equation described in the experimental section and the data from the standard curve, the R_L for a 2-ppb sample was 1.24. Because this number was greater than the observed ion ratio, the assay was negative for the presence of IP in this particular sample of turkey muscle. The result from the HIP ion chromatogram for this same sample was quite different. The m/e 185 to m/e 188 ion ratio was 1.37. The HIP standard curve that day had a slope (±SD) of 0.51 ± 0.007 with an ion ratio intercept (±SD) of 0.16 ± 0.03 (Figure 6). Thus, the R_L value for a 2-ppb HIP sample was 1.10, and because this number was less than the observed ion ratio, the assay was positive for the presence of HIP in this sample of turkey muscle.

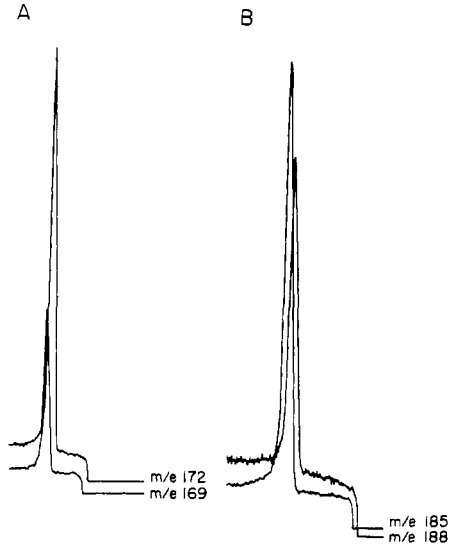

Figure 7. Ion chromatograms from the analysis of 100 g of a muscle sample from a turkey dosed with IP prior to slaughter. The tissue was analyzed for both IP (A) and HIP (B). IP-d_3 (m/e 172) and HIP-d_3 (m/e 188) were added to the sample to give each a concentration of 2 ppb. Using the standard curve shown in Figure 5, the ion intensities in ion chromatogram A suggested the presence of 0.5 ppb of IP. For this ion chromatogram, 0.6 μL, out of the available 1000 μL, was injected. Using the standard curve shown in Figure 6, the ion intensities in ion chromatogram B suggested the presence of 2.4 ppb of HIP. For this ion chromatogram, 0.6 μL out of the available 1000 μL was injected.

CONCLUSIONS

The procedure we described is ideally suited to confirm the presence of IP and HIP in turkey skin or muscle. The use of GC-NCIMS gives the procedure excellent sensitivity and specificity. The use of deuterated analogues of IP and HIP provides an accurate method to determine the recoveries and retention times of IP and HIP and to test for the presence of interfering substances.

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