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Detection of carbon-14 eluting from a capillary gas chromatograph using chemical reaction interface–mass spectrometry

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SUMMARY

We have applied a chemical reaction interface–mass spectrometric (CRIMS) technique to the detection of ^{14}C-labeled phenytoin and its metabolites in urine following separation by capillary gas chromatography. The presence of ^{14}C was followed by monitoring $^{14}CH_4$ which is produced by the chemical reaction interface from carbon when hydrogen is used as the reactant gas. Chromatograms showing ^{14}C are obtained by measuring $^{14}CH_4$ at m/z 18.034 with a resolution of 1800. The chemical reaction interface permits symmetrical, narrow chromatographic peaks to be obtained. Detection limits of 6.3 Bq/ml (1.26 Bq on-column which is equivalent to detection of 187 pg) of a diethylated ^{14}C-labeled metabolite was detected at a signal-to-noise ratio of 3. The analysis was determined to be linear and similar detection limits were obtained following analysis of phenytoin-spiked urine or a phenytoin solution. Radiocarbon detection is not completely selective because nitrogen-containing compounds present at greater than 250 ng on-column are detected as NH_4^+. However, such interferences are infrequently of analytical importance because the capacity limits of CRIMS are 500–1000 ng. Furthermore, these interferences are readily discovered in a NH_3 chromatogram which is the precursor of NH_4^+. After a 3.3-MBq dose, the major metabolites of phenytoin were detected by CRIMS.

INTRODUCTION

Incorporation of a radioisotope such as ^{14}C is intended to make a drug and its labeled metabolites unique. This is particularly useful in a study of xeno-

biotic biotransformation. Several radioisotope detectors have been utilized with gas chromatographic (GC) methods [1–8]. The presence of a radiolabel in a gas chromatogram leads to further investigation of these fractions which contain the label, thereby greatly simplifying the investigation. Because of the high volume of radiodetectors, the chromatographic resolution, however, is usually poor. A capillary GC–chemical reaction interface–mass spectrometric (CRIMS) technique [9–12] has been shown to selectively detect ^{13}C-, ^{15}N- and ^{2}H-labeled phenytoin [9] and its metabolites in a sensitive, reproducible and structure-independent manner. We wished to apply CRIMS to the detection of a ^{14}C-labeled drug and its metabolites following high-resolution capillary GC.

EXPERIMENTAL

Apparatus

The design and operation of a capillary gas chromatograph–chemical reaction interface–mass spectrometer has been described previously [9–12]. A chemical reaction interface is a microwave-powered device which decomposes a complex molecule in the presence of helium. The addition of a reactant gas forms stable products which reflect the elemental composition of the original analyte and which are detected by a conventional mass spectrometer. A Model 3300 gas chromatograph (Varian Assoc., Walnut Creek, CA, U.S.A.) which was equipped with a Varian Model 1075 split/splitless injector was used with a 30 m × 0.25 mm I.D., 0.25 μm film thickness, DB-5 capillary column (J & W Scientific, Folsom, CA, U.S.A.). Ultrapure carrier-grade helium and carrier-grade hydrogen (Air Products, Allentown, PA, U.S.A.) were fitted with high-purity stainless-steel regulators (Matheson Gas Products, Secaucus, NJ, U.S.A.). The helium was operated with a flow-rate of 1 or 2 ml/min. The injector temperature was 265°C and the initial column temperature was 80°C. Following injection of 2 μl of sample, the chromatograph was programmed to 130°C at 50°C/min at the start of the run and then at 10, 4 and 3°C/min to 165, 220 and 250°C, respectively. The analysis began at 5 min into the column temperature program when the solvent had eluted.

This work used a DuPont 21-492 mass spectrometer which was pumped at the source housing by a 15-cm NRC diffusion pump and a 5-cm CVC diffusion pump at the analyzer. The mass spectrometer was equipped with an Analytical Specialties (St. Louis, MO, U.S.A.) Model 667 power supply which had a maximum output of 5000 V for acceleration and ±500 V for the electric sector with rapid voltage switching and settling times of less than 5 ms which allowed multiple ion recording. This device is also available from Vacumetrics (Ventura, CA, U.S.A.). The power supply was controlled by a Teknivent (Maryland Heights, MO, U.S.A.) data acquisition interface, its accompanying software and an IBM XT Model 286 computer.

Operation of the chemical reaction interface

The pressure in the chemical reaction interface and the quantity of helium are important parameters for successful operation. The quantity of helium required to sustain the plasma was 1–2 ml/min. This instrument has no provision for measuring source or interface pressure, so its actual value is unknown. We adjusted flow to the chemical reaction interface by monitoring the indicated helium pressure in the source housing. Using a Penning (CVC Products, Rochester, NY, U.S.A.) ionization gauge, a pressure of approximately $3 \cdot 10^{-5}$ Torr for the DuPont 492 mass spectrometer was measured. In addition to the carrier gas flow, the pressure in the chemical reaction interface was also dependent upon the length and I.D. of the transfer line. The day-to-day operation of the chemical reaction interface is very reproducible once these parameters are optimized. A 10% increase of pressure was detected upon addition of hydrogen (carrier grade, Air Products) to the chemical reaction interface. This quantity of hydrogen produces a pale red plasma in the chemical reaction interface. It should be noted that compounds present at much greater than 1 μg on-column should be avoided as they may alter the chemical reaction interface chemistry causing erroneous results (further loss of selectivity) or malfunction (soot formation or quenching of the plasma).

Detection of ^{14}C

Detection of ^{14}C was achieved using hydrogen as the reactant gas and monitoring ^{14}CH$_4$ at m/z 18.034 with a resolution of 1800 so that ^{14}CH$_4$ was resolved from H$_2$O at m/z 18.011 and from ^{15}NH$_3$ at m/z 18.024. A non-selective chromatogram of carbon-containing compounds can be generated by monitoring CH$_4$ at m/z 16.031 at a resolution of 1800 which is sufficient to separate this mass from NH$_2$ at m/z 16.019 and O at m/z 15.995. A chromatogram which detects ammonia can be generated by monitoring NH$_3$ at m/z 17.027 or its isotopic variant, ^{15}NH$_3$ at m/z 18.024 with a resolution of 1800.

For all studies, the settling times of the mass spectrometer were 1, 1 and 5 ms for mass changes of 0.1, 1 and >1 a.m.u., respectively, with one sample obtained per peak. For the analysis of ^{12}CH$_4$, m/z 16.031 was integrated for 14 ms, averaged thirty times for a total cycle time of 0.42 s. For the analysis of NH$_3$, m/z 17.027 was integrated for 30 ms and averaged fifteen times for a total cycle time of 0.45 s. A total cycle time of 0.5 s for the analysis of ^{14}C-labeled metabolites was performed with an integration time of 165 ms and averaged three times. For the linearity studies, the total cycle time was 0.5 s with an integration time of 100 ms and averaged five times.

Methods

A male beagle dog was placed in a metabolic cage 24 h prior to administration of ^{14}C-labeled phenytoin. During this time control urine was collected, filtered and frozen until analyzed. A 400-μg amount of 4-^{14}C-labeled phenytoin (3.3 MBq, New England Nuclear, Boston, MA, U.S.A., 2.06 GBq/mmol), present in 1 ml of ethanol, was administered piggy-back through a Y-tube into a saline

solution which was flowing at a rate of 1 ml/min. The phenytoin solution was injected slowly over a period of 1 min. Urine was collected for 48 h following the injection of labeled phenytoin, filtered and frozen until analysis. To a 10-ml aliquot, which was 1% of the total volume of urine collected, [2-^{14}C]-phenobarbital (Cambridge Isotope Labs., Woburn, MA, U.S.A., 2.06 GBq/mmol) was added as an internal standard to produce a concentration of 1177 Bq/ml. The samples were hydrolyzed, extracted and derivatized by the method described previously [9]. Toluene (100 μl) was added to the derivatized residue.

Two additional experiments were carried out to validate these measurements. In one experiment, ^{14}C-labeled phenytoin was added to 10 ml of control urine to produce final concentrations of ^{14}C-labeled phenytoin from 17.7 to 722 Bq/ml. Each urine sample was hydrolyzed, extracted and derivatized as described above. This evaluated the linearity of detection of ^{14}C-labeled phenytoin. In further experiment, a set of samples containing pure ^{14}C-labeled phenytoin between 178 and 1780 Bq was derivatized. This evaluated whether detection of ^{14}C was affected by the matrix from which it was derived. In each of these experiments, 5.88 kBq of [^{14}C]-phenobarbital was added to each preparation as an internal standard. The ratio of areas under the peaks for phenytoin to the internal standard in the chromatograms generated by monitoring m/z 18.034 was determined and plotted against the calculated ratio of Bq of phenytoin to Bq of internal standard. The data collected from each experiment were pooled and a regression analysis was performed using a weighting of $1/y^2$.

To determine the disintegrations per second present in the chromatogram, radioactivity in each sample was also measured with a Beckman Model LS-3801 liquid scintillation counter. In general, the quantity of radiolabel detected represented less than 50% of the amount of radiolabel added due to losses which occurred during the extraction, hydrolysis and derivatization of each sample. These data were then manipulated to represent what would have been observed with on-line flow radioactive monitoring. To preserve chromatographic integrity from our peaks having 6-s half widths, a flow counter could retain a sample for no more than 1 s. Therefore, the total disintegrations per second in a peak should be divided by its half-width to express the peak height which would have been observed in a 100% efficient detector. The right hand y-axis of each ^{14}CH$_4$ chromatogram is labeled with this value. Most radio-GC methods have residence times which result in measuring ^{14}C passing through a detector at intervals of substantially greater than 1 s [1–8].

To evaluate the detection limit of ^{14}C-labeled phenytoin metabolites using CRIMS, the smallest peak present in a ^{14}C chromatogram was determined from the ratio of the area of this peak to the area of the internal standard, which contains a known quantity of radiolabel.

RESULTS

The characteristic high chromatographic resolution obtained using a capillary column was exhibited by symmetric peak widths at half height of approximately 6 s. This high resolution was preserved by CRIMS because of the low apparent dead volume of the ceramic tube, 0.82 cm³, which is under sub-atmospheric pressure.

Carbon-containing molecules are converted to $^{12}CH_4$ and detected at m/z 16.031. Fig. 1 shows this non-selective chromatogram of urine from a dog administered ^{14}C-labeled phenytoin. This is similar to what would be obtained by a flame ionization GC detector. A chromatogram of $^{14}CH_4$ at m/z 18.034 with a resolution of 1800 is shown in Fig. 2. Note that the majority of peaks
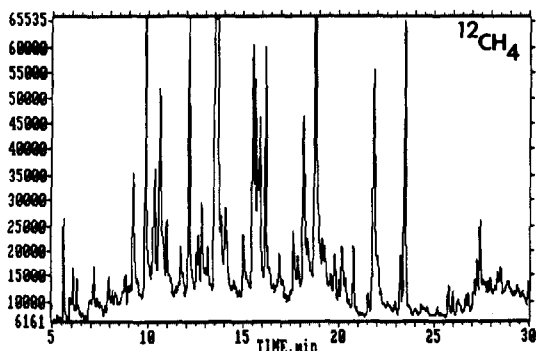

Fig. 1. Non-selective capillary GC–CRIMS profile obtained by monitoring $^{12}CH_4$ at m/z 16.031 for a derivatized extract of urine from a dog receiving 3.3 MBq of [4-^{14}C]phenytoin. The left side of the y-axis represents ion intensity and the bottom axis is time.
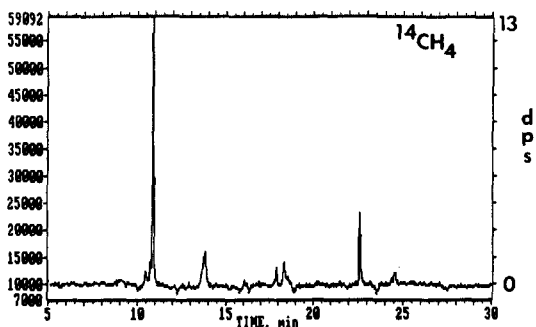

Fig. 2. Capillary GC–CRIMS profile obtained by monitoring $^{14}CH_4$ at m/z 18.034 for a derivatized extract of urine from a dog receiving 3.3 MBq of [4-^{14}C]phenytoin. To this urine, 11.7 KBq of [2-^{14}C]phenobarbital (t_R 11 min) was added as an internal standard. The left side of the y-axis represents ion intensity, the right side represents dps calculated from the measured dpm (4754 dpm) of the internal standard and the bottom axis represents time.

present in the non-selective carbon chromatogram are not detected in the ^{14}C chromatogram. The peak shoulder at retention time (t_R) 24.4 min represents 1.26 Bq or 187 pg of a ^{14}C-labeled metabolite. The signal-to-noise ratio for this peak is 3 indicating that a metabolite present at a lower level may not be detected. However, the selectivity is not perfect.

The peaks at t_R 10.5, 13.7, 16 and 18.4 min in the ^{14}CH$_4^+$ chromatogram are a result of production of NH$_4^+$ formed by ion molecule reactions from the NH$_3$ produced by large amounts of nitrogen-containing compounds. This type of interference is identified from peaks which were detected in a chromatogram of NH$_3$ at m/z 17.027. By comparison of the two chromatograms, peaks present in the ^{14}CH$_4$ chromatogram which were likely produced from nitrogen can be determined; the remaining peaks are presumed enriched with ^{14}C. These interfering peaks are also differentiated by their unusual shape.

Analysis of ^{14}CH$_4$ at a higher resolution than 1800 did not result in the disappearance of nitrogen-containing compounds but did decrease sensitivity. This was a result of the presence of ^{14}NH$_4^+$ at m/z 18.034 which could not be resolved from ^{14}CH$_4$. This species was produced in the ion source from ammonia despite conditions of the instrument which minimized the production of NH$_4^+$, i.e. high repeller voltage. We found that a nitrogen-containing compound will be detected at m/z 18.034 when present at more than 250 ng on-column. Because the capacity limit of CRIMS is between 500 and 1000 ng [9], little analytically useful data are affected by this process. Although phenytoin is a nitrogenous compound, the amount analyzed in each experiment was less than 10 ng on-column. Therefore the nitrogen content of phenytoin does not contribute to the detection of ^{14}C-labeled phenytoin.

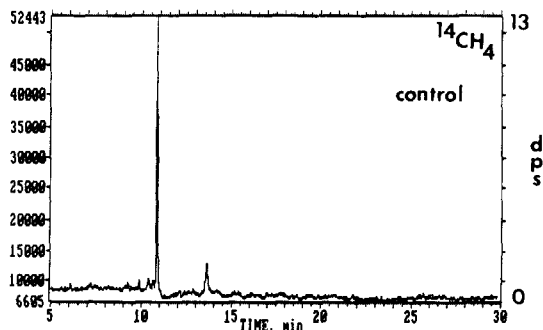The selectivity of the analysis is further demonstrated in Fig. 3 which is a

Fig. 3. Capillary GC–CRIMS profile obtained by monitoring ^{14}CH$_4$ at m/z 18.034 for a derivatized extract of control urine obtained prior to drug administration. To this urine, 11.7 KBq of [2-^{14}C]phenobarbital (t_R 11 min) was added as an internal standard. The left side of the y-axis represents ion intensity, the right side represents dps calculated from the measured dpm (4754 dpm) of the internal standard and the bottom axis represents time.

^{14}C chromatogram of urine collected from the dog prior to drug administration. The peak at t_R 13.7 min was due to production of NH_4^+. Despite the presence of more than 125 peaks (as shown in Fig. 1), few peaks are detected other than phenobarbital, the internal standard.

In Fig. 4 the time scale is expanded between 18 and 28 min. While in many of the chromatograms presented, the apparent resolution is low because of the limitations in plotting the time axis, a true representation of the chromatography is present in Fig. 4 in which the x-axis expansion was made. It shows
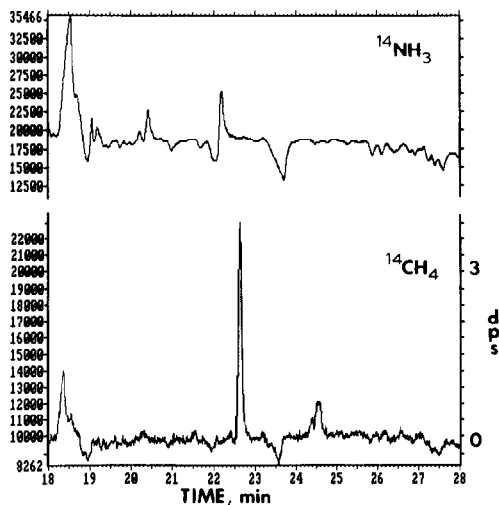

Fig. 4. Expansion of x-axis and y-axis and overlay of a chromatogram representing detection of nitrogen as NH_3 at m/z 17.027 (top) and $^{14}CH_4$ at m/z 18.034 (bottom) from Fig. 2. The peak shoulder at t_R 25.3 min represents on-column detection of 1.26 Bq which is equivalent to 187 pg of a ^{14}C-labeled phenytoin metabolite.
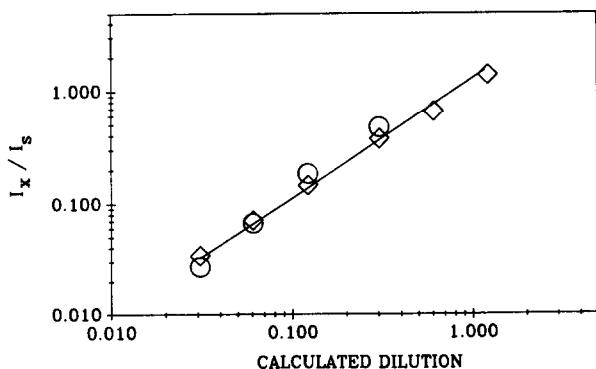

Fig. 5. Plot of measured versus calculated ratio of Bq of ^{14}C-labeled phenytoin to ^{14}C-labeled phenobarbital (internal standard) added to urine (diamonds) or when derivatized directly (circles).

an overlay of a chromatogram of representing nitrogen detected at NH_3 at m/z 17.027 with the ^{14}C chromatogram shown in Fig. 2. The one peak in the ^{14}C chromatogram which is not radiolabeled elutes at a t_R of 18.4 min as determined by detection of a peak in the chromatogram showing NH_3. The peaks at 22.7, 24.4 and 24.6 min contain ^{14}C and coincide with retention times of phenytoin metabolites (m-hydroxyphenytoin, unknown and p-hydroxyphenytoin, respectively) detected in our previous study using stable isotopes [9].

The linearity of ^{14}C detection was determined by an analysis of ^{14}C-labeled phenytoin added to urine, or phenytoin derivatized directly (Fig. 5). A plot was generated of the ratio of the ^{14}C signal for phenytoin to the internal standard (^{14}C-labeled phenobarbital) versus the calculated ratio of Bq added. The correlation coefficient of the pooled data set was 0.97 with a slope of 1.26. This indicates that detection of ^{14}C is linear. As little as 40 pci (200 pg) on-column of ^{14}C-labeled phenytoin was detected either for phenytoin added to urine or phenytoin which was derivatized directly.

DISCUSSION

The major metabolites of phenytoin, m- and p-hydroxyphenytoin, were detected in the ^{14}C chromatogram. The retention times for each of these compounds coincided with their expected retention times identified in a previous study [9]. The analysis of ^{14}C was not completely selective due to the presence of NH_4^+ produced for large quantities of nitrogen-containing compounds. Our analysis becomes problematic only when a ^{14}C-labeled compound coelutes with a nitrogen-containing compound present at 250 ng on-column. The use of an in-line cold trap for ammonia prior to detection by the mass spectrometer might improve selectivity substantially. Although other reactant gases, such as nitrogen and oxygen, and other reaction products containing ^{14}C have been explored, no other scheme yielded this level of detection. We recognize that other radio-GC methods are totally selective. Resolution, however, is inadequate. CRIMS offers high resolution with detection of ^{14}C-labeled material present at less than 3.7 Bq on-column.

We examined other ^{14}C-containing CRIMS products such as $^{14}CO_2$ at m/z 46 produced when oxygen or sulfur dioxide was used as the reactant gas. Also present at this mass was the NO_2 and $^{12}C^{16}O^{18}O$ produced by CRIMS from nitrogen and carbon-containing compounds, respectively. An enriched isotope chromatogram [10] of $^{14}CO_2$ at m/z 46, generated by subtracting the natural abundance of $^{12}C^{16}O^{18}O$, was not completely selective due to the presence of NO_2 produced from nitrogen-containing compounds. Its sensitivity was also compromised by the large subtraction needed to obtain an enriched ^{14}C chromatogram. Since m/z 18.034 at 1800 resolution was a clearer channel, it was preferred.

We have demonstrated previously that CRIMS can detect ^{13}C-, ^{15}N-, and

²H-labeled metabolites in a sensitive and highly selective manner [9]. These stable isotopes are also detected in a structure-independent manner, a feature previously only achievable for radiolabeled compounds. Studies which use stable isotopes do not have the hazards which are generally associated with use of ^{14}C. These studies with CRIMS may make their use even more favorably considered as alternatives to using ^{14}C.

CONCLUSIONS

The data presented are the first applications of GC–CRIMS for radioisotopic analyses in drug metabolism. The coupling of a capillary GC column to a chemical reaction interface–mass spectrometer provides superior resolution for ^{14}C detection as compared to a proportional flow counter. This method detects the presence of ^{14}C in a compound regardless of its structure with on-column detection well below 1 dps at peak maximum. Although the method is not completely selective, a ^{14}C-labeled compound can be distinguished from non-radiolabeled interferences present in a chromatogram. We believe that the analysis of phenytoin and its metabolites in urine, using only a simple purification and derivatization scheme, was a stringent test of the applicability of the method.

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