Novel Approach to Performing Metabolite Identification in Drug Metabolism

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Abstract

A novel online method is developed, using liquid chromatography (LC)–accurate radioisotope counting dynamic-flow (ARC) coupled with a radioactivity detector and mass spectrometer, for metabolite identification in drug discovery and development. This method offers the advantages of improved sensitivity for detecting radiolabeled drugs as well as streamlining the process of identifying and characterizing metabolites. For the purposes of evaluating this method, in vitro human liver microsomal incubations with [^{14}C]dextromethorphan are conducted. Online separation and identification of [^{14}C]dextromethorphan metabolites are achieved without intensive sample preparation, concentration, or fraction collection. Mass spectrometric analysis identified and characterized the metabolites of dextromethorphan formed by *N*- and *O*-dealkylation, correlated well with previously published results. Chromatographic peaks for [^{14}C]dextromethorphan and its metabolites are collected online, then infused for extended periods of time at a flow rate of 10 µL/min while maintaining the column pressure. The continuous analytical signal input allowed acquisition of a higher order of multistage fragmentation for both major and minor metabolites. The multistage MS fragmentation pattern obtained for the metabolites allowed defining the sites of metabolism for dextromethorphan. Further evaluations of this method are also conducted using a [^{14}C]compound A to check the linearity and sensitivity of the dynamic-flow method. The R^2 value is 0.996 for the dynamic-flow method between 50 and 600 disintegrations per minute (dpm); the limit of detection for LC–ARC is 20 dpm, which is approximately 10 times more sensitive than conventional continuous-flow radioactivity detection techniques. The overall results suggest that the combination of LC–ARC with radioactivity detection and mass spectrometry has great potential as a powerful tool for enhancing the sensitivity of radioisotope measurement in metabolite identification studies during drug discovery and development.

Introduction

The use of radioactively labeled drugs is important in many studies of drug metabolism, such as absorption, bioavailability, distribution, biotransformation, excretion, metabolite identification, and other pharmacokinetic studies (1–4). The radioactive isotopes ^{14}C or tritium [^{3}H] are typically used for labeling a given drug. Liquid chromatography (LC) separation works well with radioactive labeling, allowing high resolution, quantitative detection of unknown metabolites, and real-time monitoring by connecting the LC-radioactivity detector outlet to mass spectrometry (MS). MS detector interfaces are useful to generate data for structural elucidation of metabolites and biotransformation pathways for an administered drug. Structure elucidation of metabolites is one of the most challenging tasks in drug metabolism studies. This becomes evident when working with metabolites with a low concentration in biological matrices, such as blood plasma. Identification of metabolites usually involves several steps: separation, detection, obtaining structural information through MS, and NMR. For unknown metabolites, radiolabels (e.g., ^{14}C or ^{3}H) are usually used for differentiating the metabolite peaks from biological components. The detection sensitivity of both radioactivity and MS is an important key to successfully identify and characterize metabolites (5–8).

There has been great progress in the emerging science of detecting trace amounts of radiolabeled or non-radiolabeled drugs and their metabolites (9–11). The availability of these technologies should have a dramatic impact on drug discovery and development for metabolite profiling studies (12–14). Regulatory policy dictates that exposure to administered radioactivity be held as low as possible in most studies, which demands much greater sensitivity of the radioactivity detector to be able to detect metabolites (1). It has been reported that a microplate scintillation counter combined with capillary LC can be used to enhance sensitivity by eluent fractionation and subsequent offline counting. The limitations with this method are that the sample must be completely dry before counting, that any volatile compounds are likely to be lost, and there is the potential for apolar compounds to adsorb on the surface of the plate. Accelerator MS has been applied for the detection of ^{14}C-labeled triazine metabolites in urine. These techniques have the limitations of time-consuming sample preparation, high analysis costs, and the inability to elucidate metabolite structure. Recently, a detection method combining online LC–accurate radioisotope counting dynamic-flow (ARC) stop-flow coupled

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with a radioactivity detector and MS for metabolite identification was developed (13,14). This method has the advantages of enhanced sensitivity for radioisotope measurement and easy interface with the MS, allowing acquisition of mass spectrometric data online. There is a disadvantage to this method, and that is that the run time is longer than actual LC run time.

Herein a novel detection method combining online LC–ARC dynamic-flow coupled with a radioactivity detector and MS. Using this method, the total run time remains similar to conventional radio-LC. A technique to collect the peaks online and then infuse them to the MS is also developed, which allows the acquisition of a higher order of multistage fragmentation for both major and minor metabolites. The multistage MS fragmentation pattern obtained for the metabolites enabled the determination of the sites of metabolism.

Experimental

Chemicals and materials

[^{14}C]dextromethorphan was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Figure 1 shows the chemical structure of dextromethorphan. Human (pooled) liver microsomes (pooled male lot number 0510007 and female lot number 0410044) were obtained from a commercial source (Xenotech LLC, Kansas City, KS). Magnesium chloride ($MgCl_2$), potassium phosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADPH), and ethylenediaminetetraacetic acid (EDTA) were also from Sigma (St. Louis, MO). All other chemicals were reagent grade. The analytical column was a Prodigy C18 (250 × 2.0 mm, 5 µm), obtained from Phenomenex Inc. (Torrance, CA).

LC–ARC

LC was performed on the Surveyor LC system (Thermo Quest, CA) coupled with a β-RAM radiometric detector (IN/US Systems Inc., Tampa, FL), accurate radioisotope counting, XFlow, and ARC data system (AIM Research Company, Hochessin, DE). The flow cells and cocktail were obtained from AIM Research Company. The ARC flow cell is designed specifically for accurate radioisotope counting in radio-LC applications. The AD cocktail is compatible with LC solvents; there is no gel formation; mixing is better to form a homogeneous sample, and there is no luminescence. The flow rate from the analytical LC column was at 0.25 mL/min and split through a T-piece with online check valve. The β-RAM radiometric detector was equipped with a radiochemical liquid cell (ARC flow cell, 1000 µL) using the stop flow AD cocktail (obtained from AIM Research, Hochessin, DE); the flow rate of the cocktail was 0.75 mL/min. LC–ARC software was designed to have full control over the radioactivity detector and LC with full flexibility of the integration system.

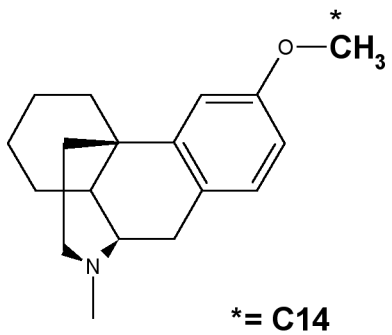

Figure 1. Chemical structure of [^{14}C]dextromethorphan, the test compound used for this study.

LC–MS analysis

A general method was used to perform LC–MS and LC–MS–MS experiments. LC–MS was carried out by coupling a Surveyor LC system to a Finnigan linear ion trap MS, LTQ (Thermo Quest, CA). The LTQ ion trap MS was equipped with an electrospray ionization source (ESI). For this study, the instrument was operated in ESI positive ion mode. The ESI source was operated at 4.5 kV and with a heated capillary temperature of 250°C. The sheath gas flow (Nitrogen) was set to 30. For MS–MS experiments, the normalized collision energy used was 50, and the collision gas was helium. LC was carried out using a Prodigy C18 column (250 × 2.0 mm, 5 µm). The LC column was maintained at 40°C. The gradient program was carried out in 32 min with water containing 5mM ammonium acetate (mobile phase A) and methanol–0.1% formic acid (mobile phase B) at 0.25 mL/min. Both solvents were degassed online. The gradient program was conducted as follows: initial 75% A; held for 2 min at 75% A; linear gradient for 13 min to 50% A; held for 10 min at 50% A; linear gradient for 1 min to 2% A; hold for 1 min at 2% A; linear gradient for 1 min to 75% A; and equilibration for 4 min at 75% A.

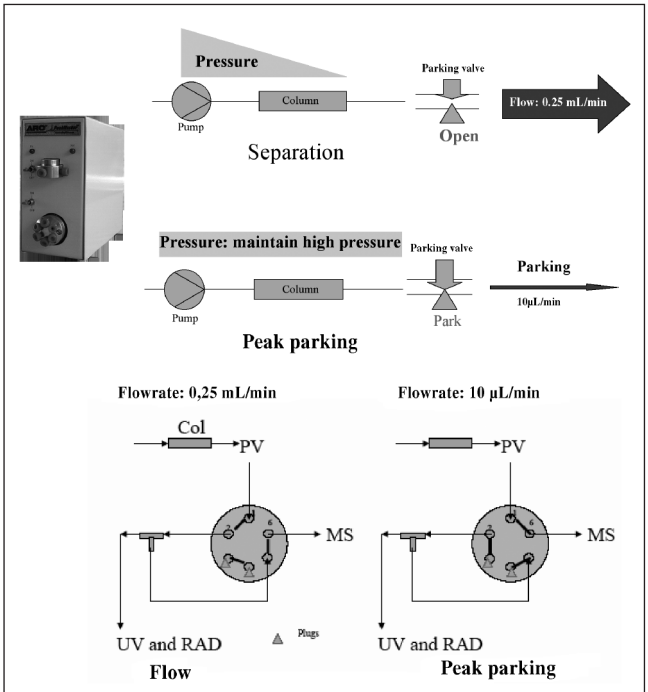

Figure 2. Hardware schematic diagram of the XFlow system. PV = Parking Valve; UV = Ultravoilet detector; RAD = radioactivity detector; MS = mass spectrometer; Col = HPLC column.

Microsomal incubations

Metabolites formed from [^{14}C]dextromethorphan were generated using human liver microsomes for up to a 60 min incubation period. The dimethyl sulfoxide concentration in the incubations was 0.1% (v/v). Microsomal incubations were performed in the presence of a nicotinamide adenine dinucleotide phosphate (NAPDH)-generating system composed of $MgCl_2$ (3mM), NADPH (1mM), glucose-6-phosphate (5mM), EDTA (1mM), and glucose-6-phosphate dehydrogenase (1 Unit/mL) in potassium phosphate buffer (50mM); all concentrations are relative to the final incubation volume. The pH of the final incubation mixture was approximately 7.4 (15,16). [^{14}C]dextromethorphan was diluted to obtain a final incubation concentration of 20µM. Final protein concentrations were 0.5 mg/mL. Incubations were conducted at 37 ± 1°C with samples taken at 0 and 60 min. The reaction was quenched by addition of two volumes of acetonitrile. The suspension then was vortexed for 1 min and centrifuged at 1300 rpm for 10 min. The samples then were loaded onto an LC column for LC–MS–MS and radioisotope analysis.

Results and Discussion

XFlow system

Figure 2 shows the hardware schematic diagram of the XFlow system. This system is simple and requires no custom-made hardware. The XFlow system uses ARC's specially designed cells and is operated under the ARC Data System, which controls the entire radio-LC system, including LC and the radioactivity detector. When interfaced with LC–MS, the XFlow system enhances the detection of low level radioactive peaks, while increasing the flexibility and productivity of MS testing. The total run time remains similar to conventional radio-LC.

Online peak collection and multistage mass experiments

It is important to note that this system can be used to collect the peaks of interest online (peak parking), then infuse them for extended periods of time at flow rates as low as 1 µL/min while maintaining the column pressure. The peaks of interest can be triggered by radioactivity signals, UV signals, or specified retention time, allowing analysts to use direct infusion with a low flow rate, with only one run, and a small sample size. The peak parking feature allows sustained analytical signal input (infusion fashion), which allows any or all of the following experiments to be done as desired: optimizing mass spectrometric condition, tuning for any individual metabolite, tuning for both positive and negative polarities, and optimizing collision energy. Also, it allows the operator to conduct multistage mass experiments (MS^n), automatically acquire ion mass data, compare isotopic mass spectra, and perform neutral loss tests. This method provides the capability of identifying the structures of unknown metabolites or impurities, again requiring a limited sample amount and a single run. This is a significant improvement over an offline fraction collector, which may lose volatile compounds during the fraction collection process. Because this method retains these compounds for analysis, it greatly expands the ability to characterize and identify metabolites of a given compound, which in turn is of significant benefit to analysts.

Sensitivity and linearity of dynamic-flow

Radioisotope counting is unaffected by LC solvent composition. There is no need for a quenching curve. The flow cells and cocktail can have an effect on background, efficiency, counting time, memory effect, and statistics. The ARC flow cells are designed specifically for accurate radioisotope counting in radio-LC applications. They give lower dead volume, virtually no memory effects, and lower background. The stop flow AD cocktail is designed to improve the cocktail–eluent ratio, which in turn produces higher counting efficiency. The limit of quantitation (LOQ) is defined here as the analyte level that gives a signal-to-noise ratio greater than 10. Limit of detection (LOD) is defined here as the analyte level that gives a signal-to-noise ratio greater than 3. LOQ is the lowest standard that consistently meets the described criteria. Results were calculated using peak area, and calibration curve was generated using a weighted (equal) linear regression. In order to check the performance of dynamic-flow in terms of linearity and sensitivity, [^{14}C]compound A was analyzed. Figure 3 shows that the linearity of [^{14}C]compound A (the time points were taken on the same day); R^2 was 0.9960 for the dynamic-flow method between 50 and 600 dpm, with a limit of detection of approximately 20 dpm. The dynamic-flow method makes it possible to generate high-resolution radio-chromatograms in actual LC run time.

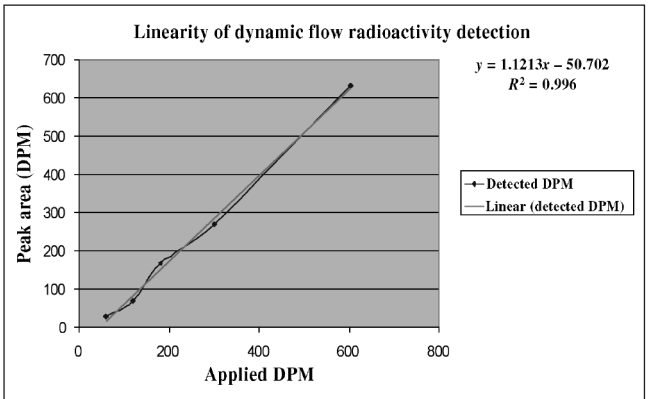

Figure 3. Linearity of dynamic-flow method at low concentration.

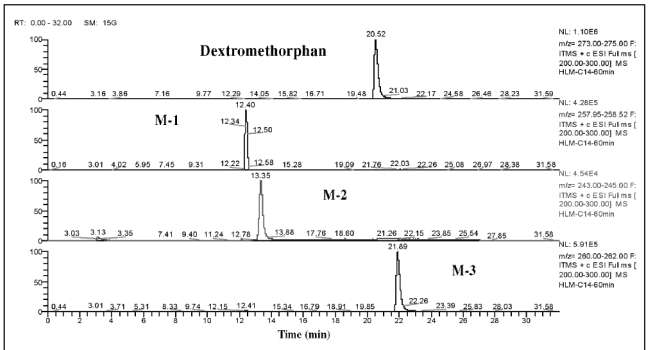

Figure 4. HPLC–MS chromatograms of [^{14}C]dextromethorphan following incubation with pooled human liver microsomes for 60 min in the presence of NADPH showing M-1, M-2, M-3, and nonmetabolized dextromethorphan.

Generation of metabolites

For the purpose of evaluating this system, in vitro human liver microsomal (HLM) incubations were performed with [^{14}C]dextromethorphan, a semisynthetic narcotic cough-suppressing ingredient in a variety of over-the-counter cold and cough medications. The in vitro metabolism of this compound is well understood. In order to evaluate the LC–ARC method for metabolite identification purposes, dextromethorphan metabolites were generated using human liver microsomes over a 60-min incubation period. Metabolites were separated by LC followed by radioisotope measurement of dextromethorphan using ARC with a IN/US radiochemical detector, and metabolite elucidation was performed with MS. Metabolic products formed during in vitro microsomal incubations were separated using a Prodigy C18 column (250 × 2.0 mm, 5 µm) maintained at 40°C. The gradient program was carried out in 32 min with water containing 5mM ammonium acetate (mobile phase A) and methanol–0.1% formic acid (mobile phase

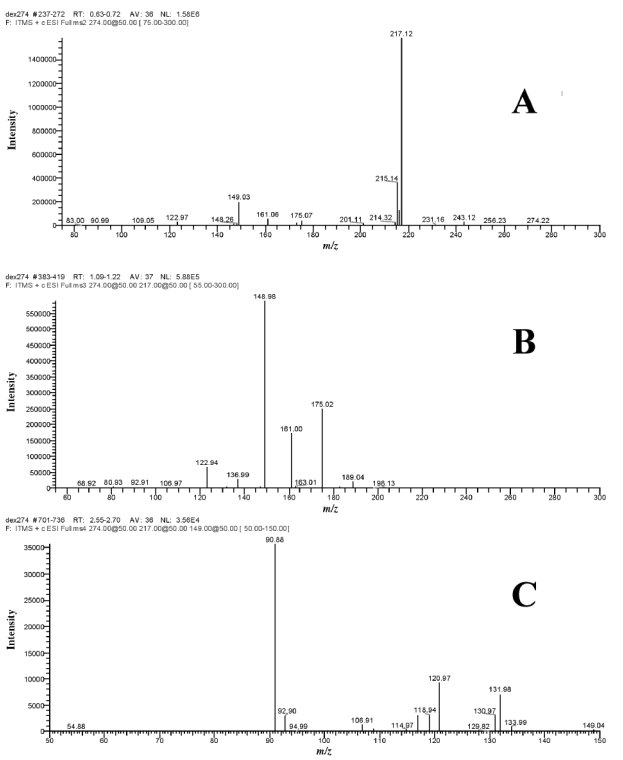

Figure 5. Representative LC–MS spectra of [M+H]$^+$ *m/z* 274, nonmetabolized [^{14}C]dextromethorphan, MS^2 (A), MS^3 (B), and MS^4 (C) following incubation of [^{14}C]dextromethorphan with human liver microsomes.

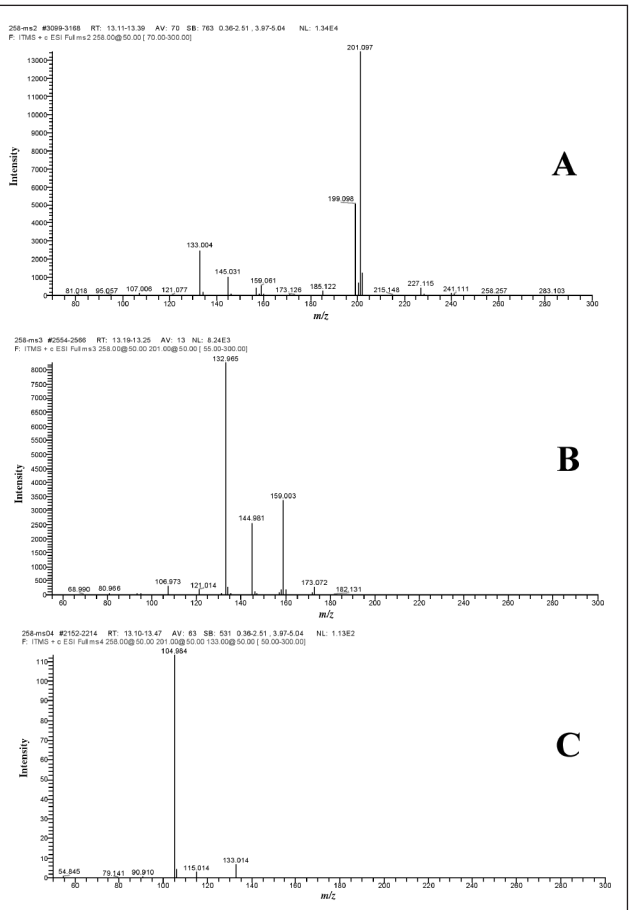

Figure 7. Representative LC–MS spectra of [M+H]$^+$ *m/z* 258, M-1, MS^2 (A), MS^3 (B), and MS^4 (C) following incubation of [^{14}C]dextromethorphan with human liver microsomes.

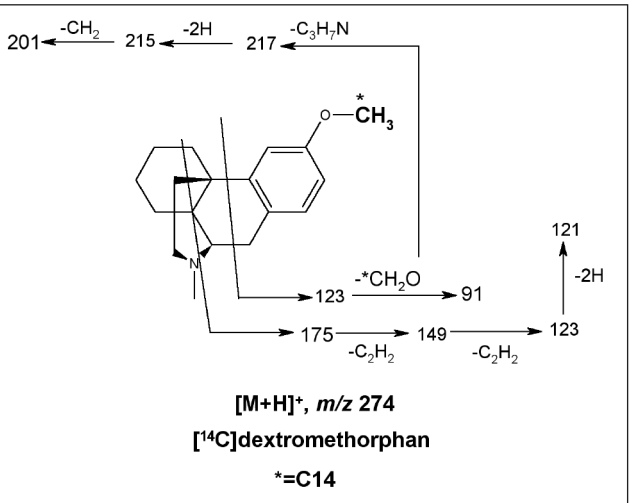

Figure 6. Proposed MS–MS fragmentations of [^{14}C]dextromethorphan.

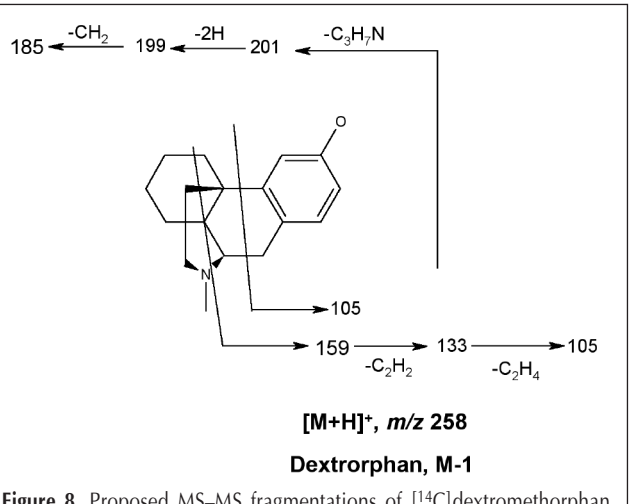

Figure 8. Proposed MS–MS fragmentations of [^{14}C]dextromethorphan metabolite, M-1.

B) at 0.25 mL/min. The online separation and identification of dextromethorphan metabolites did not require intensive sample preparation, concentration, or fraction collection.

Online LC–ARC coupled with MS for characterization of dextromethorphan and metabolites

Dextromethorphan and its metabolites were separated using the general LC–MS method described in the experimental section. Mass spectra were acquired online. Following incubation of [^{14}C]dextromethorphan with human liver microsomes for 60 min in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), three metabolites were detected: M-1, M-2, and M-3, along with the parent drug. The retention times of dextromethorphan and these metabolites were between 12 and 23 min with excellent separation efficiency. Metabolites M-1, M-2, and M-3 have retention times of 12.4, 13.3, and 22.9 min, respectively. Figure 4 shows the LC–MS chromatograms of dextromethorphan following incubation with HLM for 60 min in the presence of NADPH. The LC-radio chromatogram shows that M-1 and M-2 did not have radioactive peaks, though M-3 and the parent, dextromethorphan, did. The full-scan mass spectra for M-1, M-2, and M-3 revealed protonated molecular ions $[M+H]^+$ at *m/z* 258, 244, and 260, suggesting that M-1 lost one methyl that contained C14, M-2 lost two methyl groups (one of which contained C14), and M-3 lost one methyl group.

The structures of dextromethorphan and metabolites were elucidated by LTQ-MS–MS analysis. These peaks were collected online and then infused at a flow rate of 10 µL/min; MS^2, MS^3, and MS^4 were performed with sufficient time to examine the MS data and decide on the next fragment ions to be used for metabolite characterizations. Separate runs were performed for MS^2, MS^3, and MS^4, each without collecting the peaks; the results were similar. Figure 5 represents LC–MS spectra of nonmetabolized [^{14}C]dextromethorphan $[M+H]^+$ at *m/z* 274: MS^2 (A), MS^3 (B), and MS^4 (C). The fragment ions are *m/z* 217, 215, 201, 175, 149, 123, 121, and 91. The proposed fragmentations for non-

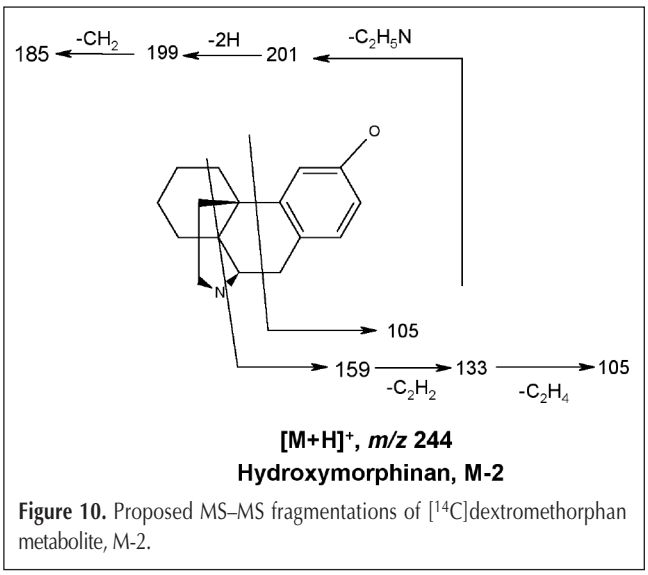

Figure 10. Proposed MS–MS fragmentations of [^{14}C]dextromethorphan metabolite, M-2.

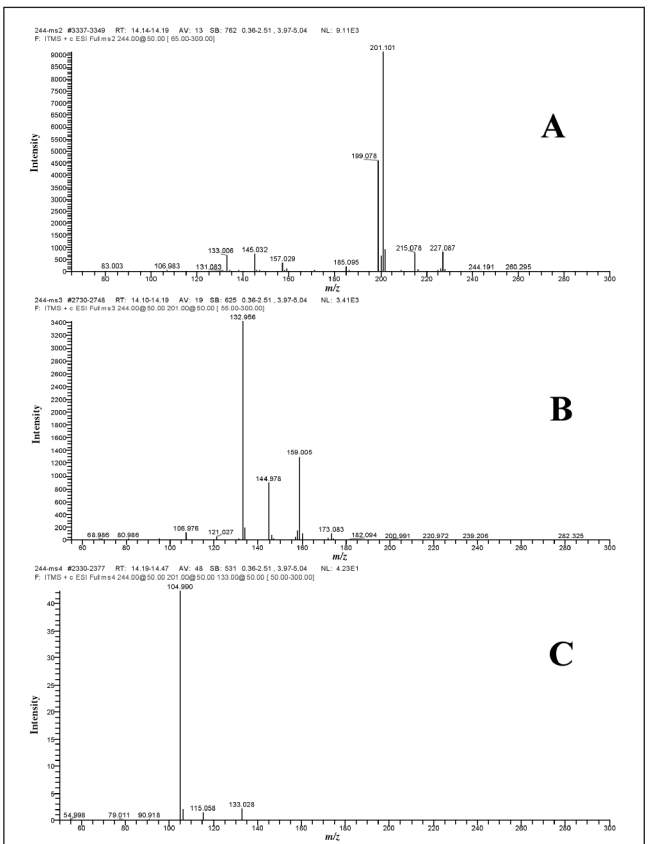

Figure 9. Representative LC–MS spectra of $[M+H]^+$ *m/z* 244, M-2, MS^2 (A), MS^3 (B), and MS^4 (C) following incubation of [^{14}C]dextromethorphan with human liver microsomes.

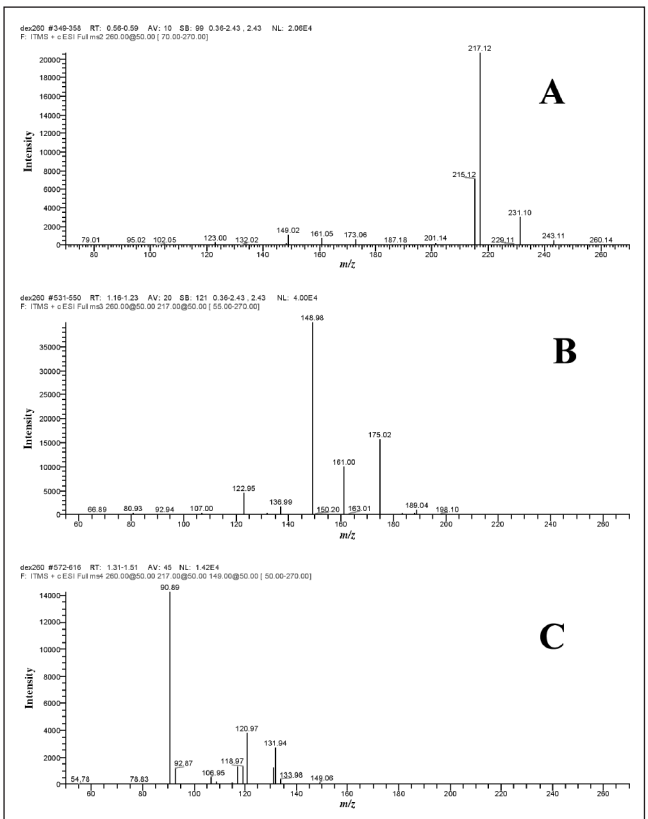

Figure 11. Representative LC–MS spectra of $[M+H]^+$ *m/z* 260, M-3, MS^2 (A), MS^3 (B), and MS^4 (C) following incubation of [^{14}C]dextromethorphan with human liver microsomes.

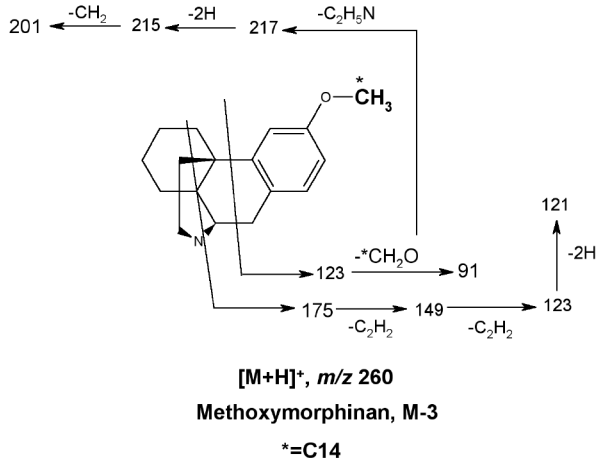

Figure 12. Proposed MS–MS fragmentations of [14C]dextromethorphan metabolite, M-3

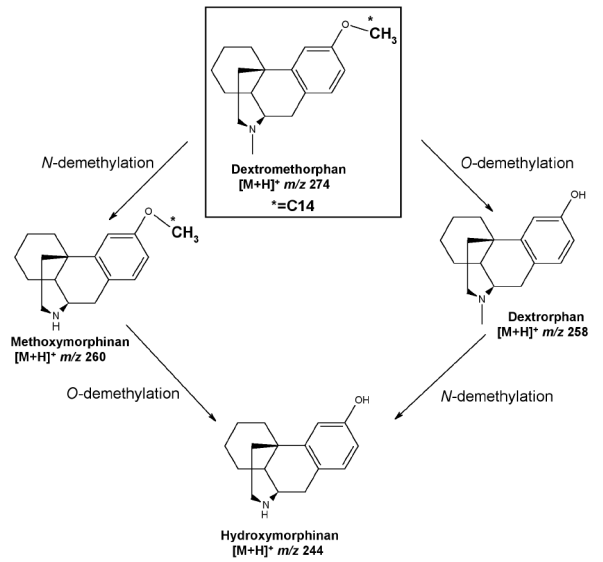

Figure 13. Proposed metabolic pathways of dextromethorphan in human hepatic microsomal incubations based on LC–ARC-MS–MS data.

metabolized [^{14}C]dextromethorphan is shown in Figure 6. Figure 7 represents LC–MS spectra of dextrorphan M-1 $[M+H]^+$ at *m/z* 258: MS^2 (A), MS^3 (B), and MS^4 (C). The fragment ions are *m/z* 201, 199, 185, 159, 133, and 105. Comparison of the proposed MS^4 fragmentation for M-1 with the parent drug fragmentation suggests that *N*-dealkylation took place on the dextromethorphan molecule. Figure 8 shows proposed MS^4 fragmentations of [^{14}C]dextromethorphan metabolite M-1. Figure 9 represents LC–MS spectra of hydroxymorphinan M-2 $[M+H]^+$ at *m/z* 244: MS^2 (A), MS^3 (B), and MS^4 (C). The fragment ions are *m/z* 201, 199, 185, 159, 133, and 105. Comparison of the proposed MS^4 fragmentation for M-2 with the parent drug fragmentation suggests that *N*- and *O*-dealkylation took place on the dextromethorphan molecule. Figure 10 shows the proposed MS^4 fragmentations of [^{14}C]dextromethorphan metabolite M-2. Figure 11 represents LC–MS spectra of methoxymorphinan M-3 $[M+H]^+$ *m/z* 260: MS^2 (A), MS^3 (B), and MS^4 (C). The fragment ions are *m/z* 217, 215, 201, 175, 149, 123, 121, and 91. The proposed MS^4 fragmentation for M-3 compared with the parent drug fragmentation suggests that *O*-dealkylation took place on the dextromethorphan molecule. Figure 12 shows proposed MS^4 fragmentations of the [^{14}C]dextromethorphan metabolite M-3. Figure 13 shows the proposed metabolic pathways of dextromethorphan in hepatic microsomal incubations. Mass spectrometric analysis showed the presence of dextromethorphan metabolites formed by *N*- and *O*-dealkylation, correlating with previously published results.

Conclusion

A novel detection method combining online LC–ARC dynamic-flow, radioactivity detector, and MS was developed. One of the major benefits of this method is that it is up to 10 times more sensitive in detecting ^{14}C peaks than conventional continuous-flow radioactivity detection techniques. Another clear advantage for this method is that the peak parking feature allows sustained analytical signal input (infusion fashion), which allows a wide variety of MS experiments for metabolite characterization to be done as desired. This method enhances the resolution of radiochromatograms and is able to measure volatile metabolites. Another advantage to this system is the easy interface with the MS, which allows acquisition of mass spectrometric data online. The method gives accurate column recovery and quantitation of low-level radioactivity and high resolution throughout the run. An important safety benefit is that by using this method, injection size has been reduced, thereby decreasing potential exposure to radioactivity and reducing the amount of radioactive wastes. This smaller injection size, and the capability to perform many tests on a single run, also reduces both time and expense significantly. Furthermore, it is easier because it reduces manual operations. This study showed that impressive progress has been made in the technology of radioisotope counting and metabolite characterization in drug metabolism using LC–ARC. The overall results suggest that the combination of LC–ARC dynamic-flow with radioactivity detection and MS has great potential as a powerful tool for radioisotope measurement in metabolite identification studies during drug discovery and development.

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