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On-Line Liquid Chromatography-Accurate Radioisotope Counting Coupled with a Radioactivity Detector and Mass Spectrometer for Metabolite Identification in Drug Discovery and Development

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A novel detection method combining on-line liquid chromatography-accurate radioisotope counting (LC-ARC, advanced stop flow controller) coupled with a radioactivity detector and mass spectrometer has been developed. One of the major benefits of this method is that this system enhances the sensitivity of radioisotope measurement for metabolite identification in drug metabolism studies. Another advantage to this system is the easy interface with the mass spectrometer, which allows acquisition of mass spectrometric data on-line. For purposes of evaluating this system, in vitro microsomal incubations with [^{3}H]propranolol were conducted. On-line separation and identification of [^{3}H]propranolol metabolites was achieved without intensive sample preparation, concentration, or fraction collection. Mass spectrometric analysis showed the presence of propranolol major metabolites formed by hydroxylation, correlating with previously published results. Further evaluations of this system also were conducted using two ^{14}C compounds, which are herein labeled X and Y. As our results show, ^{14}C peaks were detected down to 6 cpm, which is ∼20 times more sensitive than commercially available flow through radioactivity detectors. The overall results suggest that the combination of LC-ARC with radioactivity detection and mass spectrometry has great potential as a powerful tool for improving the sensitivity of radioisotope measurement in metabolite identification studies during drug discovery and development.

Radioactively labeled drugs have played important roles in many studies of drug metabolism such as absorption, bioavailability, distribution, biotransformation, excretion, metabolite identification, and other pharmacokinetic studies.[1−5] In combination with high-performance liquid chromatography (HPLC) separation, radioactive labeling allows for high resolution, quantitative detection of unknown metabolites, and real-time monitoring by connecting the HPLC-radioactivity detector outlet to other detectors such as ultraviolet, fluorescence, or mass spectrometers (MS). Such detector interfaces work well to generate data for structure elucidation of metabolites and biotransformation pathways for an administered drug.[6−8] In most studies, the radioactive isotopes ^{14}C or tritium are used for labeling of a given drug. Because they are inexpensive and easy to synthesize, ^{3}H-labeled compounds are widely used radioisotopes in biological tracing.[9,10]

To detect low metabolite concentrations formed in vitro or in vivo, the following characteristics are desirable for an HPLC-radioactivity detector: high sensitivity, low noise, on-line monitoring, easy shielding, small dimensions, easy displacement of a flow cell to another with a different volume, no absorption of samples in a flow cell, and portability. Regulatory policy dictates that exposure to administered radioactivity be kept as low as possible in most studies, which demands much greater sensitivity of the radioactivity detector. Efforts have been made to improve the detection limit of radioisotopes. Some commercially available radioactivity detectors produce fairly sensitive measurements and have been used in metabolite analysis of radiotracers. However, they still need further improvement to obtain better sensitivity and avoid intensive, time-consuming sample preparation and concentration.

As valuable as radiolabeled compounds are in metabolite identification, detection methods still need further improvements in sensitivity to be able to detect low levels of metabolite. One of the biggest challenges facing radiolabeled metabolite identification

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is the lack of a sensitive, on-line method for accurate radioisotope detection in radio-HPLC. Current radio-LC gives a limit of detection of ~300 cpm for ^{14}C using a liquid cell, which is not sensitive enough for quantitation of low-level radioactivity. To improve the limits of detection, fraction collection for off-line liquid scintillation counting is conducted to identify metabolites and determine column recovery. This fraction collection (FC) method involves many undesirable processes such as intensive manual operation and potential user exposure to radioactivity. Furthermore, the FC method cannot detect volatile metabolites and often gives lower recovery.

Alternative methods to on-line LC-radioactive detection have been developed. It has been reported that a microplate scintillation counter combined with capillary LC can be used to enhance sensitivity by eluent fractionation and subsequent off-line counting.[11] The limitations with this method are as follows: the sample must be completely dry before counting, 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 mass spectrometry (AMS) has been applied to the detection of ^{14}C-labeled triazine metabolites in urine.[12−14] Also, chemical reaction interface mass spectrometry (CRIMS) has been used, which involves the direct combustion of GC or LC eluents within a microwave-induced plasma chamber prior to analysis.[15−21] These techniques have the limitations of time-consuming sample preparation, high analysis costs, and inability to elucidate metabolite structure.

Herein we developed a novel on-line detection method, utilizing liquid chromatography-accurate radioisotope counting (LC-ARC) coupled with radioactivity and MS detectors, which enhances the sensitivity of radioisotope measuring for metabolite identification in drug discovery and development. We used [^3H]propranolol metabolites formed in vitro as part of our evaluation of this system. Figure 1 shows the chemical structure of [^3H]propranolol. The use of LC-ARC dramatically improved the sensitivity for ^{14}C peaks by up to 20-fold compared to conventional flow-through detection methods. The system allows us to control the limit of detection as desired. No fraction collector or time-consuming sample preparation is needed. The system gives accurate column recovery and quantification of low-level radioactivity and high resolution throughout the run.

EXPERIMENTAL SECTION

Chemicals and Materials. 1-[(1-Methylethyl)amino]-3-(1-naphthalenyloxy)-2-propanol, [^3H]propranolol, was obtained from Sigma Chemical Co. (St. Louis, MO). Figure 1 shows the chemical structure of [^3H]propranolol. Male rat (Wistar) and male human (pool) liver microsomes were obtained from a commercial source (Xenotech LLC, Kansas City, KS). Magnesium chloride, potassium phosphate, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and nicotinamide adenine dinucleotide phosphate (NADPH) were also from Sigma. All other chemicals were reagent grade. The analytical column Synergi, MAX-RP80A, (150 × 2 mm, 4 μm), was from Phenomenex (Torrance, CA).

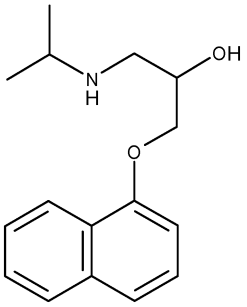

Figure 1. Chemical structure of propranolol, (1-[(1-methylethyl)-amino]-3-(1-naphthalenyloxy)-2-propanol). The test compound used for this study is labeled with tritium.

Microsomal Incubations. Metabolites formed from [^3H]-propranolol were generated using rat (Wistar, male) and human (pool, male) liver microsomes for up to a 60-min incubation period. The dimethyl sulfoxide (DMSO) concentration in the incubations was 0.2% (v/v). Microsomal incubations were performed in the presence of a NAPDH-generating system composed of $MgCl_2$ (3 mM), NADPH (1 mM), glucose 6-phosphate (5 mM), EDTA (1 mM), and 1 unit/mL glucose-6-phosphate dehydrogenase in potassium phosphate buffer (100 mM, pH 7.4); all concentrations are relative to the final incubation volume. [^3H]Propranolol was diluted to obtain a final incubation concentration of 20 μM. Final protein concentrations were 0.5 mg/mL; the initial concentrations of the liver microsomes were 20 mg/mL and they were diluted 1:1 with 0.25 M sucrose prior to the incubation. Incubations were conducted at 37 °C with samples taken at 0 and 60 min. Control samples (no NADPH) were treated as described above, but with the substitution of an equal volume of distilled water for NADPH in the NADPH-generating system to check for compound stability as well as oxidation resulting from other P450 enzymes. The reaction was quenched by addition of two volumes of acetonitrile. The suspension then was vortexed for 1 min and centrifuged at 2900 rpm for 10 min. The samples were loaded onto an HPLC column for LC−MS/MS and radioisotope analysis.

Liquid Chromatography-Accurate Radioisotope Counting. HPLC was performed on an Agilent 1100 Series Modules system (Agilent, Palo Alto, CA) coupling with Packard Radiomatic 500TR series of flow scintillation analyzers (Perkin-Elmer, Meriden, CT) and accurate radioisotope counting, advanced stop flow controller, and ARC data system (AIM Research Co., Newark, DE; www.stopflow.com). The flow cells and cocktail were obtained from AIM Research Co. The LC-ARC system utilizes advanced stop flow counting technologies to accurately detect and quantitate radio-

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isotopes. The ARC data system offers three stop flow modes: by fraction, by level, and nonstop. By fraction mode performs stop flow in a given count zone or several count zones (the area(s) where stop flow operation occurs), while by level mode performs stop flow only on the radioactive peaks and nonstop mode is the same as the traditional radio-LC, flow-through monitor where no stop flow counting is applied. The advantage of by level mode is that the total run time is shorter than the by fraction mode and longer than the nonstop mode.

The ARC flow cell is designed specifically for accurate radioisotope counting in the radio-LC application. Stop-Flow AQ cocktail is compatible with HPLC solvents, has no gel formation, and provides better mixing to form homogeneous mixtures and no luminescence. The flow rate from the analytical LC column was 0.45 mL/min and split through a T-piece (Upchurch Scientific, Inc., Oak Harbor, WA), with an on-line check valve that allowed the flow to go only in one direction. The flow to the mass spectrometer was 0.10 mL/min. The remaining flow from the splitter, 0.35 mL/min, was diverted to a Packard Radiomatic 500TR series of flow scintillation analyzer radiochemical detector equipped with a radiochemical liquid cell (ARC flow cell, 300 μL) using scintillant (Stop-Flow AQ cocktail) at a flow of 1.0 mL/min. The flow was from the radiochemical detector to the LC-ARC. LC-ARC software is designed to have full control of the radioactivity detector and HPLC with full flexibility of the integration system.

Liquid Chromatography−Mass Spectrometry Analysis. A general method was used to perform LC−MS and LC−MS/MS experiments. LC−MS was carried out by coupling a HP1100 system to a Finnigan LCQ ion trap mass spectrometer (Thermo Quest). An LCQ ion trap mass spectrometer 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 200 °C. The sheath gas flow was set to 60. For MS/MS experiments, the normalized collision energy used was 50. HPLC was carried out using a Synergi, MAX-RP80, (150 × 2 mm, 4 μm). The HPLC column was maintained at 40 °C. The gradient program was carried out in 30 min with water containing 5 mM ammonium acetate, pH 3.8 (mobile phase A) and acetonitrile (mobile phase B) at 0.45 mL/min. Both solvents were degassed on-line. The gradient program was conducted as follows: initial 90% A; hold for 2 min at 90% A; linear gradient for 20 min to 70% A; linear gradient for 1 min to 25% A; linear gradient for 1 min to 2% A; hold for 1 min at 2% A; linear gradient for 1 min to 90% A and equilibration for 4 min at 90% A.

RESULTS AND DISCUSSION

Basics of Radioactivity Detection. Radiation follows Poisson distribution and the standard deviation of radiation measurement can be expressed as follows,[22]

$$\sigma = \sqrt{C} \tag{1}$$

where C is the total count of radioisotope decay. The limit of detection (Ld) can be expressed in the following formula,

$$\mathrm{Ld} = 2.71 + 4.65\sqrt{C} \tag{2}$$

where Ld is the limit of detection in counts; when Ld is expressed as disintegrations per minute (dpm), formula 2 becomes

$$\mathrm{Ld} = \frac{271}{TE} + \frac{46.5\sqrt{B}}{TE} \tag{3}$$

where B is background (dpm), E is counting efficiency (%), and T is counting time (min). Therefore, the limit of detection in radioactivity measurement is determined by three factors: B, E, and T. Improvement in any one of these factors would improve the radioactivity detection limit.

Characteristics of LC-ARC. Measurement of radioactivity in a single vessel is different from radio-LC where a continuous flow of radioactive elute from LC is mixed with either liquid or solid scintillant to generate photon signals which are detected in photomultiplier tubes. In addition to the factors (B, E, T) mentioned above, there are at least two more important factors that affect radio-LC results: memory effect and statistical analysis of counting results. Memory effect is the phenomenon where a certain percentage of radioactivity is retained in the flow cell after the peak has theoretically passed through the flow cell and is based on flow rate and peak width. Cell type, cell volume, and chemical structures of the radioactive compounds all determine the extent of the memory effect. For solid cells, the memory effect is determined by the solid scintillant used and the chemical nature of the radioactive compounds. For example, compounds containing amino group(s) tend to have a permanent/semipermanent memory effect in solid cells. Even using a liquid cell, one can find the memory effect affecting the resolution of radioactive peaks. Due to the nature of radiation, Poisson distribution statistical methodologies must be applied in order to obtain accurate detection and quantitation of radioactivity detected. This is especially true when limit of detection and column recovery in radio-LC are calculated.

Given that the above five factors have significant negative effects on radio-LC results, it should be noted that the LC-ARC system produces improvements on all five, as described below: (a**)** The statistical analysis produced by the ARC Data System allows the user to determine the limit of detection and column recovery accurately. (b) The memory effect is reduced by cleaning flow cells after each peak. (c) The background and counting efficiency are improved by using flow cells designed specifically for radioisotope counting and a better cocktail, as described below. (d) The counting time is increased by use of the stop flow, which improves the limit of detection.

Figure 2 shows the hardware schematic diagram for a stop flow controller with a radiochemical detector and mass spectrometer. This system is simple and requires no custom-made hardware.

LC-ARC Performance. The LC-ARC was evaluated for the effect of counting time on the limit of detection, solvent effect on the radioisotopes counting, flow cells and cocktail, sensitivity for ^{14}C compounds X and Y, and characterization of [^{3}H]propranolol and its metabolites after incubation of [^{3}H]propranolol in rat and human microsomes.

Effect of Counting Time on Limit of Detection. As shown in Figure 3, the limit of detection is improved with an increase in

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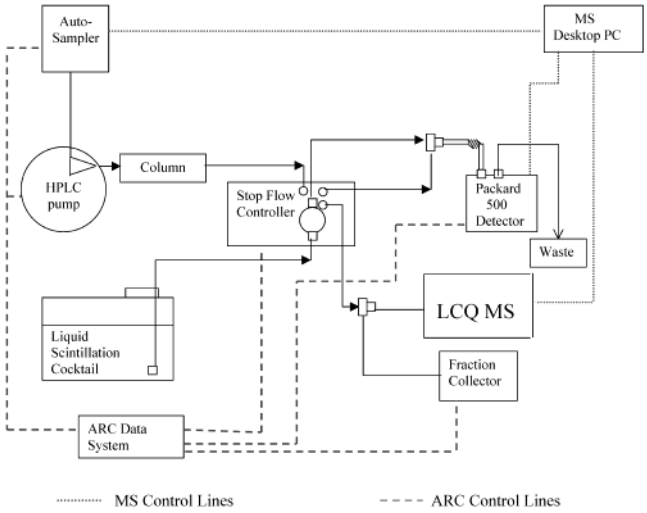

Figure 2. Hardware schematic diagram of the stop flow controller with a radiochemical detector, mass spectrometer, and fraction collector.

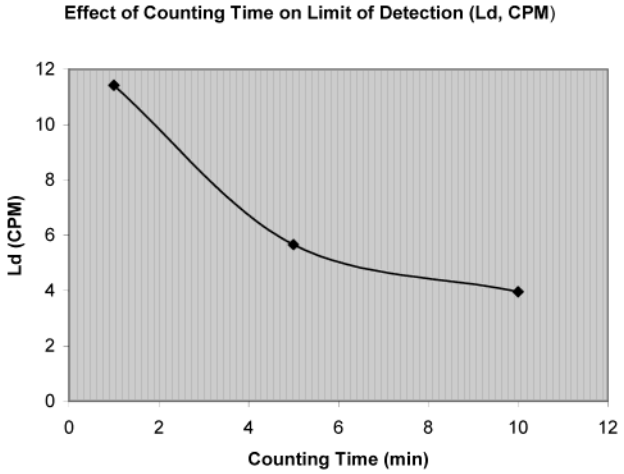

Figure 3. Effect of counting time on the limit of detection of [^{14}C]-compound Y.

the counting time. Longer counting times give better sensitivity, as predicted by eq 3. Our results, based on 1-, 5-, and 10-min counting times, were 11.4, 6.1, and 3.9 cpm, respectively. The retention times were not affected by the counting times (<0.1% relative standard deviation). The precision of the LC-ARC method was verified by analyzing the same sample over time, showing great consistency in retention times and peak areas.[23a] The LC-ARC data system allows the user to control the desired limit of detection by choosing different stop flow modes and counting times. The degree of improvement on limit of detection is not significant above 5 min of counting time.

Effect of Solvent Composition, Flow Cells, and Cocktail on Radioisotope Counting. Radioisotope counting is unaffected by LC solvent composition.[23a] 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,

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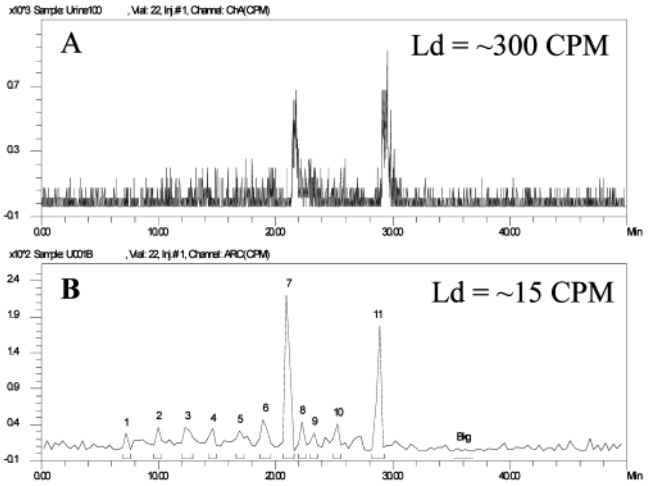

Figure 4. Comparison between (A) nonstop flow and (B) stop flow (urine sample of [^{14}C]compound X; conditions: fraction size 10 s and counting time 1 min).

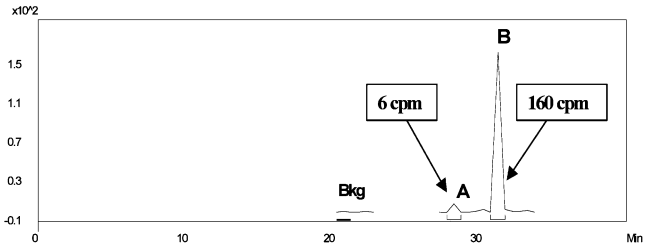

Figure 5. HPLC-ARC chromatogram showing [^{14}C]compound Y detected at the 6 cpm level with Ld of 4 cpm, (A) compound Y and (B) impurity (conditions: fraction size 10 s and counting time 2 min).

virtually no memory effects, and lower background. The stop flow AQ cocktail is designed to improve cocktail/eluent ratio (up to 2:1), which in turn produces higher counting efficiency.[23a]

LC-ARC Sensitivity. Figure 4 shows the comparison between (A) nonstop flow and (B) stop flow chromatograms from a urine sample of [^{14}C]compound X, with fraction size of 10 s and counting time of 1 min. The limit of detection using nonstop flow is 300 cpm, whereas the limit of detection using stop flow is 15 cpm. Figure 5 is an HPLC-ARC chromatogram showing [^{14}C]compound Y detected at the 6 cpm level with a 4 cpm limit of detection, peak A is the compound of interest and peak B is an impurity, the fraction size is 10 s, and counting time is 2 min. LC-ARC has dramatically improved the sensitivity of radioactivity detection by up to 20 times using an advanced stop flow controller compared to conventional flow-through detection methods. The disadvantage of this system is the run time can be 2−10 times longer than nonstop flow. The ARC data system can operate by fraction or by level. In fraction mode, it performs stop flow in a given count zone, while in level mode it performs stop flow only on the radioactive peaks. The advantage of using by level mode is that the total run time is shorter than when by fraction mode is used and longer than the nonstop mode.

Generation of Metabolites. 1-[(1-Methylethyl)amino]-3-(1-naphthalenyloxy)-2-propanol ([^{3}H]propranolol) is a β-adrenergic blocking agent that has been widely used in the treatment of hypertension, angina, and certain kinds of cardiac arrhythmias.

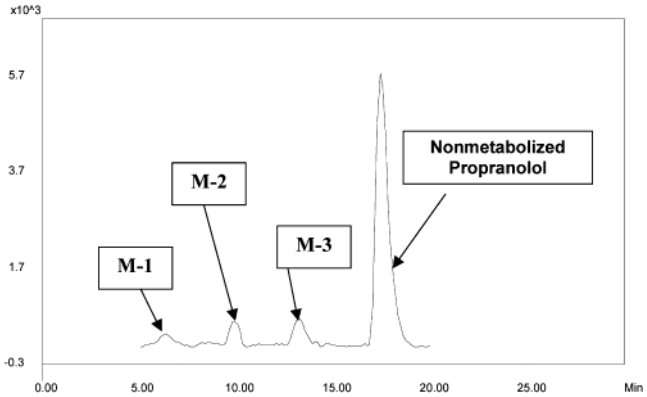

Figure 6. HPLC-ARC chromatogram of [^{3}H]propranolol following incubation with male rat microsomes for 60 min in the presence of NADPH.

The in vitro metabolism of this compound is well understood.[24−30] To evaluate the LC-ARC for metabolite identification purpose, [^{3}H]-propranolol metabolites were generated using rat (Wistar, male) and human (pool, male) liver microsomes over a 60-min incubation period. A control sample without NAPDH was also included. Metabolites were separated by HPLC, followed by radioisotope measurement of [^{3}H]propranolol using ARC with a Packard radiochemical detector, and metabolite elucidation by MS.

On-Line LC-ARC Coupled with MS for Characterization of [^{3}H]Propranolol and Metabolites. [^{3}H]Propranolol and its metabolites were separated using the general LC−MS method described in the Experimental Section. Mass spectra were acquired on-line. Following incubation of propranolol with rat and human liver microsomes for 0 and 60 min in the absence of NADPH, only the parent drug (propranolol) was detected. However, following incubation in the presence of NADPH for 60 min, three major metabolites were detected: M-1, M-2, and M-3, along with the parent drug. The retention times of propranolol and metabolites were between 5 and 20 min with excellent separation efficiency. Metabolites M-1, M-2, and M-3 have retention times of 6, 10, and 13 min, respectively. Figure 6 shows an HPLC-ARC chromatogram of [^{3}H]propranolol following incubation with male rat microsomes for 60 min in the NADPH.

Propranolol and Metabolites. The structures of propranolol and metabolites were elucidated by LCQ−MS/MS analysis. Figure 7 shows a protonated molecular ion $[M + H]^+$ at m/z 260 and 276 for (A) nonmetabolized [^{3}H]propranolol and (B) [^{3}H]propranolol M-2, respectively in human liver microsomal preparations. The full-scan mass spectrum for M-1, M-2, and M-3 revealed protonated molecular ions $[M + H]^+$ at m/z 276, 16 amu higher than the parent drug, indicating the addition of an oxygen atom. The proposed MS/MS fragmentation for nonmetabolized [^{3}H]propranolol is shown in Figure 8. The fragment ions are m/z 218, 183, 157, 116, and 98. The proposed MS/MS fragmentation for M-1, M-2, ands M-3 suggests that the oxidation occurred on the naphthalene moiety, correlating with previously published results.[27−30]

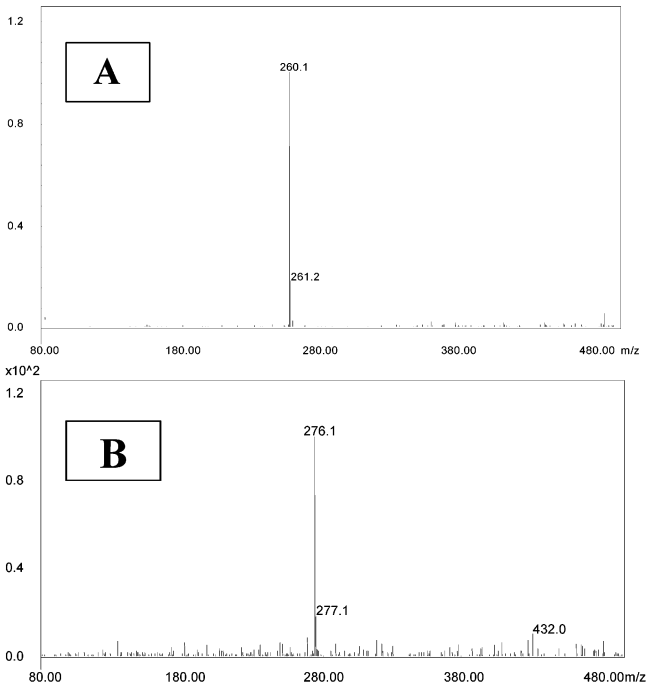

Figure 7. MS spectrum of (A) nonmetabolized [^{3}H]propranolol and (B) [^{3}H]propranolol M-2 in human liver microsomal preparations.

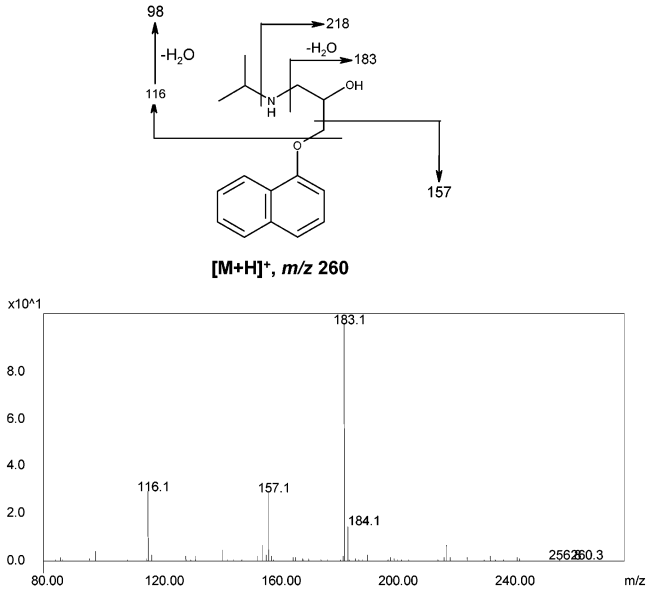

Figure 8. MS/MS spectra of nonmetabolized [^{3}H]propranolol in human liver microsomal preparations.

CONCLUSIONS

The present study shows the impressive progress that has been made in the technology of radioisotope counting in drug metabolism using LC-ARC. The LC-ARC on-line stop flow method is up to 20 times more sensitive in detecting ^{14}C peaks than commercially available flow-through radioactivity detectors. Using this method, it is possible to generate high-resolution radiochromatograms and accurately measure volatile metabolites which fraction collection methods are not even able to detect. The ability

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to acquire mass spectra on-line is also a major advancement. 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. Furthermore, it is easier because it reduces manual operations. This study was originally intended to evaluate the LC-ARC system for detection and quantification of radioactive components in metabolite identification. Since longer counting times give better sensitivity, the stop flow run time is usually longer than nonstop flow.

From our results, it appears that the LC-ARC could also be very useful in many additional areas, such as ADME studies in animals or humans, environmental studies of radiolabeled compounds, and purity determination in radiosynthesis. Further, coupling the LC-ARC system with a fraction collector for automatic isolation would provide an integrated solution enabling detection, quantitation, isolation, and structural elucidation of radioactive metabolites all in a single run.[23b]

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