Liquid Chromatography–Accurate Radioisotope Counting and Microplate Scintillation Counter Technologies in Drug Metabolism Studies

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Abstract

The present study involves an analysis of the performance of liquid chromatography (LC)–accurate radioisotope counting (ARC) and microplate scintillation counter (TopCount) technologies in drug metabolism studies. For the purpose of evaluating these systems, biological samples resulting from the metabolism of a radiolabeled [^{14}C] compound, known as compound B, are analyzed using LC–ARC and TopCount under similar high-performance LC conditions. Counting efficiency is 83% for LC–ARC, 77% for TopCount, and linearity is R^2 of 0.9998 versus 0.9984, respectively. The limit of detection for LC–ARC is 12 disintegrations per minute (dpm) with 1-min/fraction counting, yet for TopCount it is 8.7 dpm with 5-min/fraction counting. Under optimal conditions for each, the total run time of LC–ARC is approximately half that of TopCount. These results indicate that there is no significant difference between these two systems in terms of efficiency, linearity, and limit of detection. However, the LC–ARC system does not involve any manual operations, yet TopCount requires manual sample transfer and data import. This study shows that impressive progress has been made in the technology of radioisotope counting in drug metabolism using LC–ARC. This system enhances the resolution of radiochromatograms and is able to measure volatile metabolites that TopCount cannot detect at all. The ability to acquire mass spectra online is also a major advancement. The overall results suggest that the combination of LC–ARC with radioactivity detection and mass spectrometry has great potential as a powerful tool for radioisotope measurement in metabolite identification studies during drug discovery and development.

Introduction

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 (1–5). High-performance liquid chromatography (HPLC) separation works well with radioactive labeling, allowing high resolution, quantitative detection of unknown metabolites, and real-time monitoring by connecting the HPLC–radioactivity detector outlet to other detectors such as UV, fluorescence, or mass spectrometers (MS) (or both). These detector interfaces are useful to generate data for structural elucidation of metabolites and biotransformation pathways for an administered drug (6–8). The radioactive isotopes ^{14}C or tritium [^{3}H] are typically used for labeling of a given drug. Because of their low cost and ease of synthesis, ^{3}H-labeled compounds are widely used radioisotopes in biological tracing (9,10).

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. Some commercially available radioactivity detectors produce fairly sensitive measurements and have been used in metabolite analysis of radiotracers. Efforts have been made to improve the detection limit for radioisotopes; however, further improvement is still needed in order to obtain better sensitivity and avoid intensive, time-consuming sample preparation and concentration. Conventional online flow-through radioactivity detection of labeled compounds is limited by insufficient sensitivity. Current radio liquid chromatography (LC) gives a limit of detection of approximately 300 cpm for ^{14}C using liquid cell, which is not sensitive enough for the quantitation of low-level radioactivity. In order to improve the limits of detection, fraction collection for offline 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. In addition to the challenge of inadequate sensitivity, the lack of online capability is an issue in radio-labeled metabolite elucidation.

It has been reported that a microplate scintillation counter

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combined with capillary LC can be used to enhance sensitivity by eluent fractionation and subsequent offline counting (11). The limitations with this method are: (*i*) the sample must be completely dry before counting, (*ii*) any volatile compounds are likely to be lost, and (*iii*) 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 gas chromatography (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 the inability to elucidate metabolite structure.

Basics of radioactivity detection

Radiation follows Poisson distribution; the standard deviation of radiation measurement can be expressed as follows (22):

$$\sigma = \sqrt{C} \qquad \text{Eq. 1}$$

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

$$Ld = 2.71 + 4.65\sqrt{C} \qquad \text{Eq. 2}$$

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

$$Ld = \frac{271}{T \times E} + \frac{46.5\sqrt{B}}{\sqrt{T \times E}} \qquad \text{Eq. 3}$$

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 on any one of these factors would improve the radioactivity detection limit.

Characteristics of LC–accurate radioisotope counting

Measurement of radioactivity in a single vessel is different from radio-LC in which a continuous flow of radioactive elute from LC is mixed with either liquid or solid scintillant to generate photon signals that are detected in photomultiplier tubes (PMTs). In addition to the factors (B, E, and T) previously mentioned, 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 in which a certain percentage of radioactivity is retained in the flow cell after the peak has theoretically passed through the flow cell, and it is based on flow rate and peak width. Cell type, cell volume, and the chemical structures of the radioactive compounds all determine the extent of 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 permanent/semipermanent memory effect in solid cells. Even using a liquid cell, one can find the memory effect affecting the resolution of radioactive peaks. Because of 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 calculating limit of detection and column recovery in radio-LC.

Given that the mentioned five factors have significant negative effects on radio-LC results, it should be noted that the LC–accurate radioisotope counting (ARC) system produces improvements on all five, as described: (*i*) the counting time is increased by use of the stop flow, which improves the limit of detection; (*ii*) the memory effect is reduced through cleaning the flow cell by flushing with liquid scintillant after each peak; (*iii*) the background and counting efficiency are improved using a better cocktail (described later) and flow cells designed specifically for radioisotope counting. The statistical analysis produced by the ARC data system allows the user to determine the limit of detection and column recovery accurately.

Figure 1 shows the hardware schematic diagram for the stop-flow controller with a radiochemical detector and MS. This system is simple and requires no custom-made hardware. The pressure gradient was maintained throughout the run in the stop flow mode.

Herein, LC–ARC and TopCount technologies have been applied to the measurement of radiolabled drugs and their metabolites in biological samples. There is no significant difference between these two systems in terms of efficiency, linearity, and limit of detection. An online detection method utilizing LC–ARC coupled with MS and radioactivity detectors is also discussed; this system makes it possible to generate high-resolution radiochromatograms and accurately measure volatile metabolites, which TopCount cannot detect.

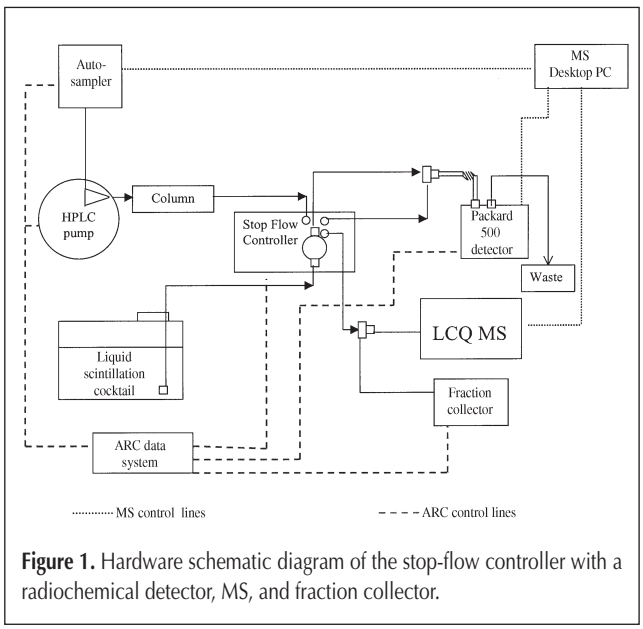

Figure 1. Hardware schematic diagram of the stop-flow controller with a radiochemical detector, MS, and fraction collector.

Experimental

Chemicals and materials

1-[(1-Methylethyl)amino]-3-(1-naphthalenyloxy)-2-propanol, [^{3}H]propranolol was obtained from Sigma Chemical Co. (St. Louis, MO). Male rat (Wistar) and male human (pool) liver microsomes were obtained from a commercial source (Xenotech LLC, Kansas City, KS). Magnesium chloride ($MgCl_2$), 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).

Microsomal incubations

Metabolites formed from [^{3}H]propranolol were generated using male rat (Wistar, male) and human (pool, male) liver microsomes up to 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 an nicotinamide adenine dinucleotide phosphate (NAPDH)-generating system composed of $MgCl_2$ (3mM), NADP+ (1mM), glucose-6-phosphate (5mM), ethylenediaminetetraacetic acid (1mM), and 1 unit/mL glucose-6-phosphate dehydrogenase in potassium phosphate buffer (100mM, pH 7.4); all concentrations are relative to the final incubation volume. [^{3}H]propranolol was diluted to obtain a final incubation concentration of 20µM. Final protein concentrations were 0.5 mg/mL, initial concentrations of the liver microsomes were 20 mg/mL, and then they were diluted 1:1 with 0.25M sucrose prior to the incubation. Incubations were conducted at 37°C with samples taken at 0 and 60 min. Control samples (no NADPH) were conducted as described previously, but with the substitution of an equal volume of distilled water for $NADP^+$ 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 then loaded into an HPLC column for LC–MS–MS and radioisotopes analysis.

LC–ARC

HPLC was performed on Agilent 1100 Series modules system (Agilent, Palo Alto, CA) coupling with Packard Radiomatic 500TR series of flow scintillation analyzers (PerkinElmer, Meriden, CT) and ARC, advanced stop flow controller, and ARC data system (AIM Research Company, Hochessin, DE). The flow cells and cocktail were obtained from AIM Research Company. The LC–ARC system utilizes advanced stop-flow counting technologies to accurately detect and quantitate radioisotopes. 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 [the area(s) where stop flow operation occurs], 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 much shorter than the by fraction mode.

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

The experimental conditions for Figure 1 were as follow: the LC–ARC system consisted of an Agilent 1100 HPLC with a binary pump, autosampler, variable wavelength detector, and PerkinElmer flow scintillation analyzer model 500TR equipped with an ARC flow cell and a StopFlow controller model B. The LC–ARC conditions were: fraction interval was set to 11.69 s with counting time of 1 min, using bylevel stop-flow mode. LC–FC–TopCount: Agilent 1100 HPLC with a fraction collector. Fractions of 12-s were collected into a 96-well plate coated with solid scintillator and then dried under vacuum. The PerkinElmer TopCount was used to count each well for 5 min. The obtained data are then imported into the ARC data system for evaluation.

LC–TopCount analyses

Urine samples coming from a man dosed with [^{14}C]compound Z were also analyzed using the LC–TopCount system. The HPLC column was maintained at room temperature. The gradient program was carried out in 140 min with water containing 3 g/L ammonium acetate (mobile phase A) and acetonitrile (mobile phase B) at 1 mL/min. Both solvents were degassed online. The gradient program was conducted as follows: initial 95% A, linear gradient for 20 min to 90% A, linear gradient for 110 min to 65% A, linear gradient for 1 s to 0% A, and hold for 10 min at 0% A and equilibration for 15 min at 95% A. Agilent 1100 LC with an Agilent 1100 fraction collector was used to collect HPLC fraction into the 96-well plates. The fraction size was set to 11.69 s. The collected eluate was then dried under a nitrogen stream. The dried plates were counted using PerkinElmer TopCount microplate 6-MTP counter. Each well was counted for 5-min. The counted results were exported into a text file that was in turn imported into the ARC data system for evaluation.

LC–MS

A general method was used to perform LC–MS and LC–MS–MS experiments. LC–MS was carried out by coupling an HP1100 system to the Finnigan LCQ ion trap MS (Thermo Quest, CA). The LCQ ion trap MS was equipped with an elec-

trospray 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. For MS–MS experiments the normalized collision energy used was 50 V. 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 5mM ammonium acetate pH 3.8 (mobile phase A) and acetonitrile (mobile phase B) at 0.45 mL/min. Both solvents were degassed online. 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

Effect of counting time on limit of detection

The limit of detection for radioisotope counting is improved with an increase in the counting time. Longer counting times give better sensitivity, as predicted by equation 3. The 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 (23). 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 at more than 5 min of counting time.

Effect of solvent composition, flow cells, and cocktail on radioisotope counting.

Radioisotope counting is unaffected by LC solvent composition (23). 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 top flow AQ cocktail is designed to improve cocktail–eluent ratio (up to 2:1), which in turn produces higher counting efficiency.[23]

Comparison between conventional flow through detection and LC–ARC

A previous comparison was made between nonstop flow and stop flow from rat urine dosed with a [^{14}C]drug, with fraction interval of 10 s and counting time 1 min (23). The limit of detection using nonstop flow was 300 cpm, whereas the limit of detection using stop flow was 15 cpm. From these results, it was clear that LC–ARC dramatically improved the sensitivity of radioactivity detection by up to 20 times using an advanced stop flow controller compared with conventional flow-through detection methods.

LC–ARC performance

Figure 2 is the HPLC–ARC chromatogram showing [^{14}C]compound A from human urine. The conditions were: 10 µL injection; stop-flow mode bylevel; Ld, 12 cpm level; fraction interval, 15 s; and counting time, 2 min. Bylevel stop-flow

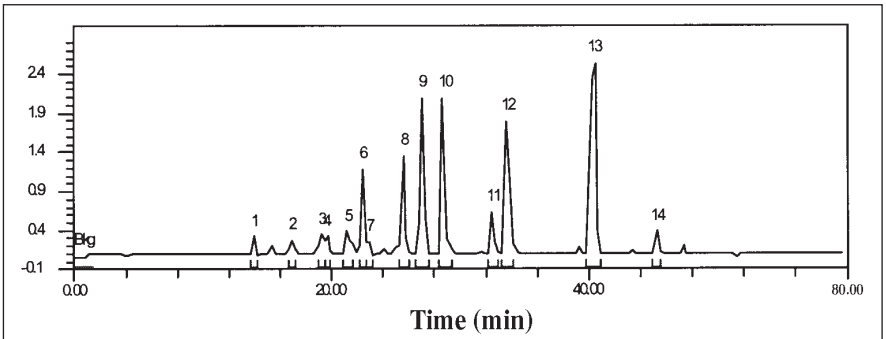

Figure 2. HPLC–ARC chromatogram showing [^{14}C]compound A and metabolites (human urine conditions: Ld, 12 cpm level; fraction interval, 15 s; and counting time, 2 min).

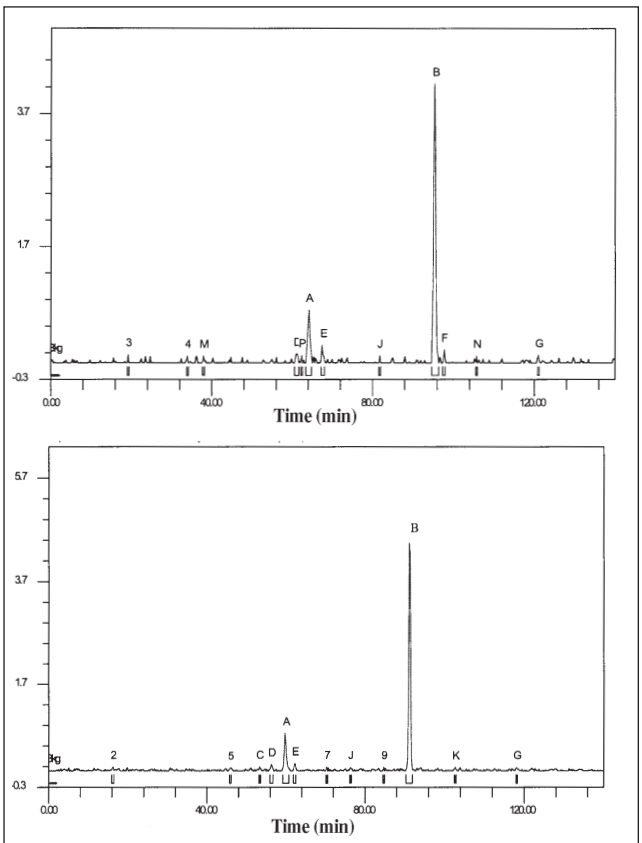

Figure 3. Comparison between (upper trace) LC–ARC and (lower trace) TopCount (plasma sample of [^{14}C]compound B, LC–ARC conditions: 10-µL injection; stop-flow mode, byLevel; fraction interval, 11.69 s; and counting time, 1 min).

mode was chosen to detect radioactive peaks only; 14 peaks were detected, 2855 dpm was injected, and 2523 dpm was recovered, giving 83% efficiency observed in this study. A disadvantage of the stop-flow system is the total run time can be up to 4 times longer than nonstop flow, depending upon whether by level or by fraction mode is used. The advantage of using level mode is that the total run time is much shorter than when using fraction mode.

Performance of LC–ARC and TopCount

In order to evaluate the performance of LC–ARC and TopCount, compound B was analyzed under similar LC conditions with TopCount and LC–ARC in its bylevel mode. In bylevel mode, LC–ARC first determines the background in the solvent front, then finds peaks and performs stop-flow measurement only for the peaks it has detected. Compound B (5-, 10-, and 30-µL injection volume) was injected under this mode. For TopCount, fractions were collected under the same conditions; additional steps and time were required for drying. Figure 3 shows the comparison between chromatograms for LC–ARC (upper trace) and TopCount (lower trace) from a plasma sample of [^{14}C]compound B (stop-flow mode, bylevel; fraction interval, 11.69 s; counting time, 1 min; and 10-µL injection volume).

Table I presents the results of our comparison of LC–ARC and TopCount. LC–ARC produced a counting efficiency of 83% vsersus 77% for TopCount, and linearity was R^2 of 0.9998 versus 0.9984 for TopCount. The limit of detection for LC–ARC was 12 dpm, but for TopCount it was 8.7 dpm. The total run time for LC–ARC was approximately 300 min with no manual operations involved. TopCount would require at least twice this time, even with a 6-PMT counter. These experiments show that the bylevel mode of the LC–ARC system can reliably detect radioactivity down to approximately 10-cpm peaks with excellent column recovery. In addition, LC–ARC is suitable for all the metabolites even if they are volatile, but TopCount cannot be used for volatile metabolites.

Table I. Comparison between LC–ARC and TopCount

	LC–ARC	LC–TopCount	
Operation			
Fr Interval	11.69 s	12 s	
Counting time	1 min	5 min	
Counting eff. (%)	83%	77%	
Counting mode	bylevel	NA	
Ld (cpm)	10 CPM	6 cpm	
Total run time	**300 min**	**3500**	for single PMT topcount
		583	for 6 PMT topcount
Fraction collection	0	150	
Manual time*	0	60	
Total time (min)	300	793	

* Including fraction setup, transfer of sample, evaporation, and data reduction.

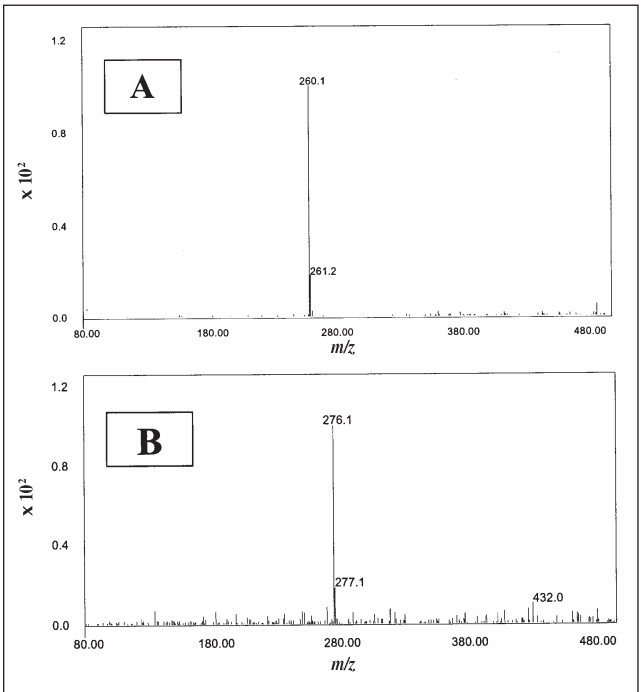

Figure 4. MS spectrum of (A) nonmetabolized [^{3}H]propranolol and (B) [^{3}H]propranolol M-2 in human liver microsomal preparations.

Online LC–ARC–MS for characterization of [^{3}H]propranolol and metabolites

Propranolol and its metabolites were separated online by HPLC followed by radioisotope measurement of [^{3}H]propranolol using ARC with a Packard radiochemical detector and metabolite elucidation by LCQ–MS–MS. Figure 4 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 spectra for M-1, M-2, and M-3 revealed protonated molecular ions $[M+H]^+$ at *m/z* 276, which was 16 amu higher than the parent drug, indicating the addition of an oxygen atom. Three monohydroxy metabolites were detected and the oxidation appeared to be on the naphthalene moiety (24–27). MS analysis showed the presence of propranolol metabolites formed by hydroxylation, correlating with previously published results.

Conclusion

The results clearly provide evidence of the continuing progress in the technology of radioisotope counting in drug metabolism. When using either LC–ARC or TopCount, sensitivity in detecting ^{14}C peaks is enhanced up to 20-fold over commercially available flow-through radioactivity detectors. In terms of counting efficiency and linearity, there is no significant difference between LC–ARC and TopCount. The total run time for LC–ARC is shorter than TopCount, particularly when using the new bylevel mode for LC–ARC. Use of LC–ARC also greatly improves the resolution of radiochromatograms and allows accurate measurement of volatile metabolites, which

fraction-collection methods such as TopCount are unable to detect. The ability of LC–ARC to acquire mass spectra online is also a major advancement, facilitating more accurate and sensitive radioisotope measurement in metabolite identification during drug discovery and development. An important safety benefit of LC–ARC is that injection interval is reduced, thereby decreasing potential exposure to radioactivity and reducing the amount of radioactive waste. Furthermore, by eliminating many of the manual operations required by TopCount, productivity is improved with the LC–ARC system. From these results, it appears that LC–ARC could be very useful in many additional areas, such as adsorption, distribution, metabolism, and excretion studies in animals or humans, environmental studies of radiolabeled compounds, and purity determination in radiosynthesis.

Acknowledgments

We would like to thank Drs. Jeffrey J. Anderson and Dave L. Ryan of DuPont, and Ms. Lefevre C. and Mr. Umbdenstock T. of Technologie Servier for their valuable discussion and suggestions.

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Manuscript accepted June 10, 2004.