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Analysis of low level radioactive metabolites in biological fluids using high-performance liquid chromatography with microplate scintillation counting: Method validation and application

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Abstract

TopCount, a microplate scintillation counter (MSC), has been recently employed as an off-line liquid radiochromatographic detector for radioactive metabolite profile analysis. The present study was undertaken to validate TopCount for metabolite profiling with respect to sensitivity, accuracy, precision and radioactivity recovery. Matrix effects of various human samples on TopCount performance and capability of MSC for volatile metabolite analysis were also investigated. TopCount had a limit of detection (LOD) of 5 DPM and a limit of quantification (LOQ) of 15 DPM for [^{14}C]-labeled compounds at a 10 min counting time. It was two-fold more sensitive than a liquid scintillation counter (LSC), and 50–100-fold more sensitive than a radioactivity flow detector (RFD). TopCount had comparable accuracy and precision to RFD, and comparable precision to LSC for determining relative abundance of metabolites. Human liver microsome incubation (up to 1 mL), plasma (up to 1 mL), urine (up to 2 mL) and feces (up to 50 mg) had no significant quenching effects on TopCount performance. Benzoic acid, a volatile metabolite, was detected by TopCount, but not by Microbeta counter after microplates were dried under vacuum. Radioactivity recovery in HPLC–MSC analysis was reliably determined using an LSC-based method. Examples of using HPLC–MSC for analysis of low levels of radioactive metabolites are presented, including determination of plasma metabolite profile, in vitro reactive metabolites trapped by [^{3}H]glutathione, and metabolite concentrations in an enzyme kinetic experiment. The data from this study strongly suggest that HPLC in combination with TopCount is a viable alternative analytical tool for detection and quantification of low levels of radioactive metabolites in biological fluids.

Keywords: Microplate scintillation counter; Radiochromatographic detection; Metabolite profiling; TopCount

1. Introduction

Radiolabeled drug candidates are routinely used in drug metabolism studies during the drug development stage [1–4]. Radiolabels, as a tracer of drug related components, not only facilitate the detection, isolation and identification of radioactive metabolites, but also allow the parent drug and its metabolites to be quantified without the use of synthetic standards. Various radiochromatographic techniques are employed for metabolite profiling in biological matrices [5] and for metabolite concentration determination in enzyme kinetic studies [6,7]. HPLC with on-line radioactivity flow detection (RFD) is the most often used radiochromatographic technique in drug metabolism. RFD provides quick radioactivity profiling results with high separation resolution [8,9]. Additionally, it enables coupling with a mass spectrometer for simultaneous metabolite identification [10–14]. However, poor analytical sensitivity of the RFD technique greatly limits its application to the samples with low levels of radioactivity. HPLC with off-line liquid scintillation counting (LSC) is often employed for the analysis of low levels or trace amounts of radioactive metabolites such as plasma metabolites [15]. Since LSC provides radioactivity measurement with DPM value, radioactivity recovery from HPLC–LSC analysis can be reliably determined. Metabolite profiling by HPLC–LSC

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consists of four steps: HPLC separation, fraction collection into test tubes, mixing with scintillation cocktail, and counting radioactivity one fraction a time. The entire process is time-consuming and labor-intensive.

Recently, microplate scintillation counting (MSC) was introduced as an off-line liquid radiochromatographic detector for radioactive metabolite profiling [16–19]. In HPLC–MSC analysis, HPLC eluent is collected into 96-well microplates, and then evaporated using a speed vacuum system. The radioactivity of residues in the 96-well plates is determined by counting up to 12 wells at a time using a microplate scintillation counter (MSC). Results from early exploratory work demonstrated that HPLC–MSC not only increases analytical throughput and sensitivity, but also reduces radioactivity waste and manual operation compared to LSC [16–19]. HPLC–MSC has become the method of choice for analysis of low level radioactive metabolites in some metabolism laboratories [17,20–24]. In addition, HPLC–MSC has been applied to metabolite identification in combination with various mass spectrometric techniques, such as determination of formation pathways and structures of secondary metabolites [25], selective identification of molecular ions of unknown metabolites in a complex biological matrix [26], and sensitive characterization of plasma [27] and tissue [28] metabolites.

Two types of MSC instruments, TopCount and MicroBeta counter, are commercially available. TopCount uses DeepWell LumaPlates®, in which yttrium silicate scintillators are deposited at the bottom of each well. MicroBeta counter uses 96-well Scintiplates® that consist of a white frame with clear wells embedded with solid scintillators. In HPLC–MSC analysis CPM values are generally utilized for calculation of relative radioactivity abundance. If counting efficiencies of HPLC eluent fractions across the entire HPLC run are consistent or vary within an accepted range, the relative abundance of radioactivity peaks calculated from CPM values would not be significantly different from those from DPM values. However, the counting efficiency of MSC could be greatly reduced by color quenching or other effects when analyzing biological samples. Consequently, the accuracy and precision of the MSC method could be affected significantly. Recently, a study observed that extracts from rat feces (equivalent to 0.4 or 1.0 g feces homogenate) or rat urine (equivalent to 1 mL urine) generated significant quenching in metabolite profiling by MicroBeta counter [22]. MSC analysis usually requires solvent evaporation using a speed vacuum device. Volatile metabolites could be lost in this process. Therefore, the use of validated method for determining radioactivity recovery is important in HPLC–MSC analysis.

Metabolite profiling and enzyme kinetic studies by liquid radiochromatographic techniques provide crucial information in support of drug development and registration. To assure quality of data from in vitro metabolism/drug interaction studies, regulatory agencies and the pharmaceutical industry suggest that these studies are to be conducted "in the spirit of GLP" [2]. The present studies were attempted to validate the HPLC-TopCount technique for metabolite profiling with respect to accuracy, precision, sensitivity, and radioactivity recovery. Matrix effects of biological samples on TopCount performance and the capability of both TopCount and MicroBeta counter for analysis of volatile metabolites were also investigated. In addition, several examples of applications of the HPLC–MSC technique to analysis of low level metabolites are presented.

2. Experimental

2.1. Chemicals and materials

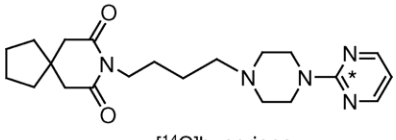
[^{14}C]-labeled buspirone (specific activity of 27 μCi/mg and a radiochemical purity >97.5%) and BMS-214778 (24.1 μCi/mg, radiochemical purity 98.8%) were synthesized at Bristol-Myers Squibb (Princeton, NJ). The structures of buspirone and BMS-214778 are shown in Fig. 1. [^{14}C]benzoic acid was generated from incubations of a [^{14}C]-labeled drug in human liver microsomes. [^{3}H]glutathione (GSH, 52.0 Ci/mmol) was from Perkin-Elmer (Boston, MA). Pooled human liver microsomes were purchased from BD Bioscience (Woburn, MA). Pooled human plasma was obtained from Lampire labs (Pipersville, PA). Pooled human feces and urine were collected from six healthy subjects. FLO^{TM} M cocktail was purchased from Perkin-Elmer (Shelton, CT). Ecolite liquid scintillation cocktail was obtained from ECN Radiochemicals (Costa Mesa, CA).

2.2. HPLC and radioactivity analysis

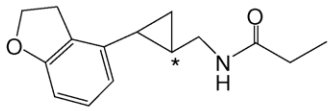
2.2.1. HPLC

HPLC analyses were performed on a Shimadzu class VP system equipped with two pumps (model LC-10AD), an autoinjector (model SIL 10AD) and a diode array detector (model SPD-MA10A). The following HPLC gradient systems were employed:

- *HPLC System I*: For analysis of buspirone metabolites, a Zorbax RX-C8 column (4.6 mm × 250 mm) and a linear stepwise gradient with Solvent A (0.01% of trifluoroacetic



[^{14}C]busprione



[^{14}C]BMS-214778

Fig. 1. Structures of [^{14}C]buspirone and [^{14}C]BMS-214778. The ^{14}C-label is indicated with an asterisk.

acid in water) and Solvent B (acetonitrile) were used [24]. Solvent B in the gradient hold at 8% (0–8 min), and then changes as follows: 40% (30 min), 90% (35 min), and 8% (40 min).

- *HPLC System II*: In the experiment of determining matrix effects of biological fluids, Zorbax RX-C8 column (4.6 mm × 250 mm) and a linear stepwise gradient with Solvent A (water) and Solvent B (methanol) were used. The flow rate was set up at 1 mL/min. Solvent B was started at 10% and then increased to 85% at 24 min. HPLC analysis was performed at a flow rate of 1 mL/min.
- *HPLC System III*: An HPLC method was developed for the analysis of [^{14}C]BMS-214778 metabolites in rat plasma and urine, which used a Zorbax RX-C18 column (4.6 mm × 250 mm) and a linear stepwise gradient with Solvent A (ammonium acetate, pH 6.1) and Solvent B (acetonitrile). Solvent B in the gradient started at 5% and then increased as follows: 7% (4 min), 20% (20 min), 35% (25 min), 40% (30 min), 58% (35 min), 80% (37 min) and maintained at 80% (37–40 min). HPLC analysis was performed at a flow rate of 1 mL/min.
- *HPLC System IV*: For analysis of benzoic acid in human liver microsomes, a YMC OD5 AQ (2.0 mm × 150 mm) column and a linear stepwise gradient with Solvent A (0.05% of trifluoroacetic acid in a mixture of 5% acetonitrile and 95% water) and Solvent B (0.05% trifluoroacetic acid in a mixture of 95% acetonitrile and 5% water). Solvent B was started at 0% and then increased as follows: 5% (10 min), 10% (20 min), 20% (30 min), 22% (35 min), 30% (40 min), 50% (50 min), 0% (55 min). HPLC analysis was performed at a flow rate of 0.25 mL/min.
- *HPLC System V*: An HPLC method was developed for the analysis of [^{3}H]GSH trapped reactive metabolites. It used a Zorbax RX-C18 column (4.6 mm × 250 mm) and a linear stepwise gradient with Solvent A (0.1% formic acid in water) and Solvent B (0.1% formic acid in acetonitrile). Solvent B in the gradient started at 0% and then increased as follows: 5% (5 min), 50% (20 min), 90% (40 min). HPLC analysis was performed at a flow rate of 1 mL/min.

2.2.2. TopCount

A standard procedure for radioactivity profiling by HPLC with TopCount was used in most experiments. Briefly, HPLC eluent was collected into 96-well microplates (Deep-Well LumaPlate®, Perkin-Elmer, Shelton, CT) at the rate of 15 s/well with a fraction collector (Gilson FC204, Middleton, WI). HPLC solvents in the mirocplates were evaporated using a speed vacuum system (SpeedVac®, RVA-400, Savant Instruments Inc., Holbrook, NY). Radioactivity (CPM value) of the residues in 96-well plates was determined with TopCount (Perkin-Elmer, Shelton, CT). Up to 12 wells were counted simultaneously with a 10 min counting time.

2.2.3. MicroBeta counter

MicroBeta counter (Perkin-Elmer, Shelton, CT) was used for analysis of a human liver microsomal incubation sample that contained [^{14}C]benzoic acid, a volatile metabolite. HPLC eluent was collected into 96-well microplates (Scintiplate®, Perkin-Elmer, Shelton, CT) in the fraction collector at a rate of 15 s/well. Before counting radioactivity, either HPLC solvents in microcplates were evaporated using a speed vacuum system or liquid scintillation cocktail (200 μL) was directly added to each well. Radioactivity (CPM value) in the 96-well microplates was determined using MicroBeta counter. Up to 12 wells were counted simultaneously with a 10 min counting time.

2.2.4. Liquid scintillation counter

HPLC eluent was collected into test tubes (30 s/tube) in the fraction collector. Each fraction was mixed with 4.5 mL of Ecolite liquid scintillation cocktail and then counted for 10 min in a liquid scintillation analyzer (Packard, Tri-Carb®, Meriden, CT).

2.2.5. Radioactivity flow detector

HPLC eluent was mixed with ULTIMA-FLO™ M cocktail at a ratio of 1:3, and then the mixture was passed through a 500 μL liquid detection cell in a radioactivity flow detector (β-Ram, IN/US System, Tampa, FL).

2.3. Metabolism experiments and sample preparations

2.3.1. Human liver microsome incubations

For determining accuracy, precision and radioactivity recovery of HPLC-TopCount method, buspirone metabolism samples were prepared. [^{14}C]buspirone (0.5–40 μM) was incubated with pooled human liver microsomes (0.2–1 mg/mL) and NADPH in sodium phosphate buffer (pH 7.4) at 37 °C. Incubations were stopped by the addition of an equal volume of ice-cold methanol. After centrifugation, supernatants were directly analyzed using various liquid radiochromatographic methods. For determining the matrix effect of human liver microsome incubations on the TopCount performance, a control HLM sample (without radiolabeled drugs) was prepared following the same procedure.

2.3.2. Metabolism study in rats

[^{14}C]BMS-214778 was intravenously administrated into two male S.D. rats (25 mg/kg, 25 μCi/kg). Plasma (1 h) and urine (0–24 h) were collected and pooled for metabolite profiling. The total radioactivity in these samples was counted by LSC. The plasma sample was pretreated by solid phase extraction (Oasis Extraction Cartridge, Waters, Milford, MA), and the urine samples were filtered before HPLC–MSC analysis.

2.3.3. Preparation of control human samples

Extracts or concentrates of biological samples (non-radioactivity) were prepared for determination of matrix effects on TopCount performance. Pooled human urine and the supernatant of a human liver microsome incubation sample

(see Section 2.3.1) were directly dried under a stream of nitrogen at room temperature. A pooled human plasma sample was loaded on a C18 Extraction Cartridge (Oasis Extraction Cartridge, Waters, Milford, MA), followed by elution with water and methanol. The methanol fraction was collected and dried under a stream of nitrogen. Pooled human fecal homogenate (feces:water, 1:1, 2 g) was extracted with 4 mL acetonitrile (30 s vortex and 10 min sonication) three times. After centrifugation supernatants were combined and dried under a stream of nitrogen.

2.4. Method validation

2.4.1. Determination of sensitivity

Limit of detection (LOD) of radioactivity analysis was calculated using Eq. (2), which was derived from Eq. (1) [29].

$$\mathrm{LD} = 2.71 + 4.65\sqrt{C} \tag{1}$$

where LD is the limit of detection expressed as the total counts and C, the total counts of background for a certain period of counting time.

C equals to the product of B (background radioactivity expressed in DPM), T (counting time) and E (counting efficiency). When the Eq. (1) is divided by T and E, it becomes Eq. (2). Eq. (2) and other similar equations are used for calculating the limit of detection of radioactive metabolite profiling [22,30].

$$\mathrm{LD} = \frac{2.71}{TE} + 4.65\sqrt{\frac{B}{TE}} \tag{2}$$

where LD is expressed in DPM.

Radioactivity counts follow the normal distribution. Therefore, the probability of the observed counts being with ±2-fold of standard deviation from the mean value is 95.5% (95.5% confidence). The standard deviation of a sample with low radioactivity near background radioactivity can be expressed as Eq. (3):

$$s = \sqrt{\frac{\mathrm{CPM_s} + \mathrm{CPM_b}}{T}} \tag{3}$$

where s is the standard deviation; $\mathrm{CPM_s}$, the count rate of the sample; $\mathrm{CPM_b}$, the count rate of the background; and T, the counting time of the sample and the background.

The net count rate of the sample equals to $\mathrm{CPM_s} - \mathrm{CPM_b}$. The value of %2$s$ is the 2s value expressed as a percentage of the net count rate of the sample. Thus, Eq. (3) can be further converted to Eq. (4) for calculating %2s values.

$$\%2s = \frac{200\sqrt{(\mathrm{CPM_a} + \mathrm{CPM_b})/T}}{\mathrm{CPM_a} - \mathrm{CPM_b}} \tag{4}$$

Limit of quantification (LOQ) of radioactivity counting can be defined as the sample net count rate when %2s value is 20%. Therefore, if radioactivity of a sample equals LOQ, 95.5% of the time the observed counts will be within the range of ±20% from the mean value. Eq. (4) is also used for calculating the minimal time to gain a desired standard deviation for counting a sample with low levels of radioactivity.

2.4.2. Determination of accuracy and precision

Metabolites of [^{14}C]buspirone in HLM were analyzed by HPLC (System I) coupled with TopCount, LSC and RFD. Percent distributions of radioactivity peaks, including buspirone and its metabolites, were determined by dividing the counts of each peak by the total counts determined in the HPLC run. Precision values of percent distribution of radioactive peaks were calculated based on five repeated analyses. Accuracy values of HPLC–MSC and HPLC–RFD for metabolite profiling were calculated by the comparison of percent distribution of each peak determined by HPLC–MSC and HPLC–RFD to the corresponding "true value" determined by HPLC–LSC.

2.4.3. Determination of matrix effects of biological samples

Extracts or concentrates of human plasma (up to 1 mL plasma), urine (up to 2 mL urine), fecal homogenates (up to 250 mg feces) and HLM incubation (up to 1 mL incubation solution, 1 mg protein/mL) were dissolved in a mixture of methanol and water (1:9), and injected into an HPLC (System II). [^{14}C]buspirone was infused (2620 DPM/min) continuously into post-column HPLC eluent by a syringe pump during the entire HPLC run. The mixture of HPLC eluent and [^{14}C]buspirone was collected into 96-well plates (15 s/well) followed by TopCount analysis (10 min counting time). The effect of biological matrices on the counting efficiency of LC-TopCount was determined by the comparison of radiochromatograms with or without injections of biological samples.

2.4.4. Determination of radioactivity recovery

A urine sample from rats after the administration of BMS-214778 (Section 2.3.2) was used for determining radioactivity recoveries in HPLC-TopCount analysis. Firstly, the urine sample (approximately 49,000 DPM) was injected and passed through an HPLC column (System I). The total HPLC eluent (40 mL) was collected into a beaker. Four aliquots (2 mL) of the eluent were counted for radioactivity by LSC (10 min counting), and additional four aliquots (2 mL) were dried using a speed vacuum system followed by radioactivity counting by LSC. Secondly, the same amount of urine was injected and the radioactivity in HPLC eluent was counted following the same procedure except that the HPLC eluent was collected without passing through the HPLC column. The determined radioactivity (DPM values) in the HPLC eluent was used for calculating column, microplate and total recoveries based on Eqs. (5)–(7), respectively.

$$\text{Column recovery}\,(\%) = \frac{\mathrm{DPM_C}}{\mathrm{DPM}} \times 100 \tag{5}$$

where DPM is the radioactivity of the HPLC eluent without passing through the HPLC column and no solvent evapora-

tion. DPM_C is the radioactivity in the HPLC eluent passing through the HPLC column and without solvent evaporation.

$$\text{Microplate recovery}\,(\%) = \frac{DPM_M}{DPM} \times 100 \quad (6)$$

where DPM_M is the radioactivity of the HPLC eluent without passing through the HPLC column and with solvent evaporation.

$$\text{Total recovery}\,(\%) = \frac{DPM_T}{DPM} \times 100 \quad (7)$$

where DPM_T is the radioactivity of the HPLC eluent passing through the HPLC column and with solvent evaporation.

2.4.5. Volatile metabolite analysis

A microsomal incubation sample containing [^{14}C]benzoic acid was injected into an HPLC (System IV). The HPLC eluent was collected into test tubes (30 s/test tube) for LSC analysis, and 96-well plates (15 s/well) for MSC analysis. The MSC analyses were conducted under three conditions: (1) use of Lumaplates followed by solvent evaporation and radioactivity counting by TopCount, (2) use of Scintiplates followed by solvent evaporation and radioactivity counting by MicroBeta counter, and (3) use of Scintiplates followed by the addition of cocktail and radioactivity counting by MicroBeta counter.

2.5. Application of MSC to analysis of low levels of radioactive metabolites

2.5.1. Plasma metabolite profiling

A plasma sample from rats dosed with [^{14}C]BMS-214778 (Section 2.3.2) was treated with solid phase extraction (Oasis Extraction Cartridge, Waters). An aliquot (approximately 493 DPM) of the plasma extract was analyzed by HPLC (System III) with TopCount (standard procedure). Percent distribution of plasma radioactivity was calculated based on the HPLC radioactivity profile.

2.5.2. Detection of GSH-trapped reactive metabolites

A non-radiolabeled drug candidate (50 μM) was incubated with human liver microsomes (1 mg/mL) and NADPH (500 μM) in the presence of a mixture of GSH (1 mM) and trace amounts of [^{3}H]GSH (1–2 μCi/mL) for 30 min. Incubation reactions were started by the addition of NADPH (500 μM) and stopped by the addition of two volumes of ice-cold methanol. After centrifugation the supernatant was directly analyzed by HPLC (System V) with TopCount (standard procedure).

2.5.3. Determination of metabolite concentrations in incubation mediums

Buspirone (0.5 μM) was incubated with HLM (0.2 mg protein/mL) for 5 min. Incubation conditions and sample treatment were the same as described above (Section 2.3.1). Metabolite profile (%distribution of radioactivity) was obtained using HPLC (System I) with TopCount (standard procedure) after 200 μL supernatant was directly injected. Concentrations of buspirone and its metabolites in the incubations were calculated based on the following equation.

$$\begin{aligned}&\text{The concentration of metabolite}\\ &= \%\text{distribution of the metabolite}\\ &\quad \times \text{initial drug concentration in incubation}\end{aligned}$$

3. Results and discussions

3.1. Sensitivity

The sensitivity (Table 1) for metabolite profiling by HPLC-TopCount and HPLC–LSC was calculated using Eqs. (2) and (4). LOD of TopCount for ^{14}C radioisotopes was 5 DPM at a 10 min counting time, which was approximately two-fold better than the LOD values of LSC. It was estimated that the LOD for RFD was from 250 to 500 DPM for the ^{14}C radioisotopes [13,23]. Therefore, TopCount was approximately 50–100-fold more sensitive than RFD. A comparison of radiodetection sensitivity of MSC with LSC and RFD is illustrated in Fig. 2. Minor metabolite peaks M1, M2, M4, M10, M13 and M14 were detected by TopCount (Fig. 2A), but not seen by RFD even when as much as four times more sample was injected (Fig. 2C). LSC was able to detect most metabolites except for a few minor metabolites. For example, M14 (15 CPM, Table 2) was not detected by the HPLC–LSC method (Fig. 2B).

LOQ is a more relevant term with respect to the sensitivity of radioactive metabolite profiling since it is defined based on not only the ratio of single to noise but also analytical accuracy. The LOQ of TopCount for [^{14}C]isotopes was 15 DPM at a 10 min counting time, which was two-fold better than the LOQ values of LSC (Table 1). As indicated in Eqs. (2) and (4), low background radioactivity (approximately 2 CPM) of TopCount is the main factor to contribute to its

Table 1
Sensitivity comparison of TopCount with LSC

Radio-detection	Background (CPM)	Counting efficiency (%)	Counting time (min)	Limit of detection[a] (DPM)	Limit of quantification[b] (DPM)
LC–LSC	25	90	10	10	31
LC–MSC (TopCount)	2	70	10	5	15

[a] Limit of detection (LOD) was calculated based Eq. (2) and the parameters used for the calculation are listed above.
[b] Limit of quantification (LOQ) was calculated based on Eq. (4) and the parameters used in the calculation are listed above.

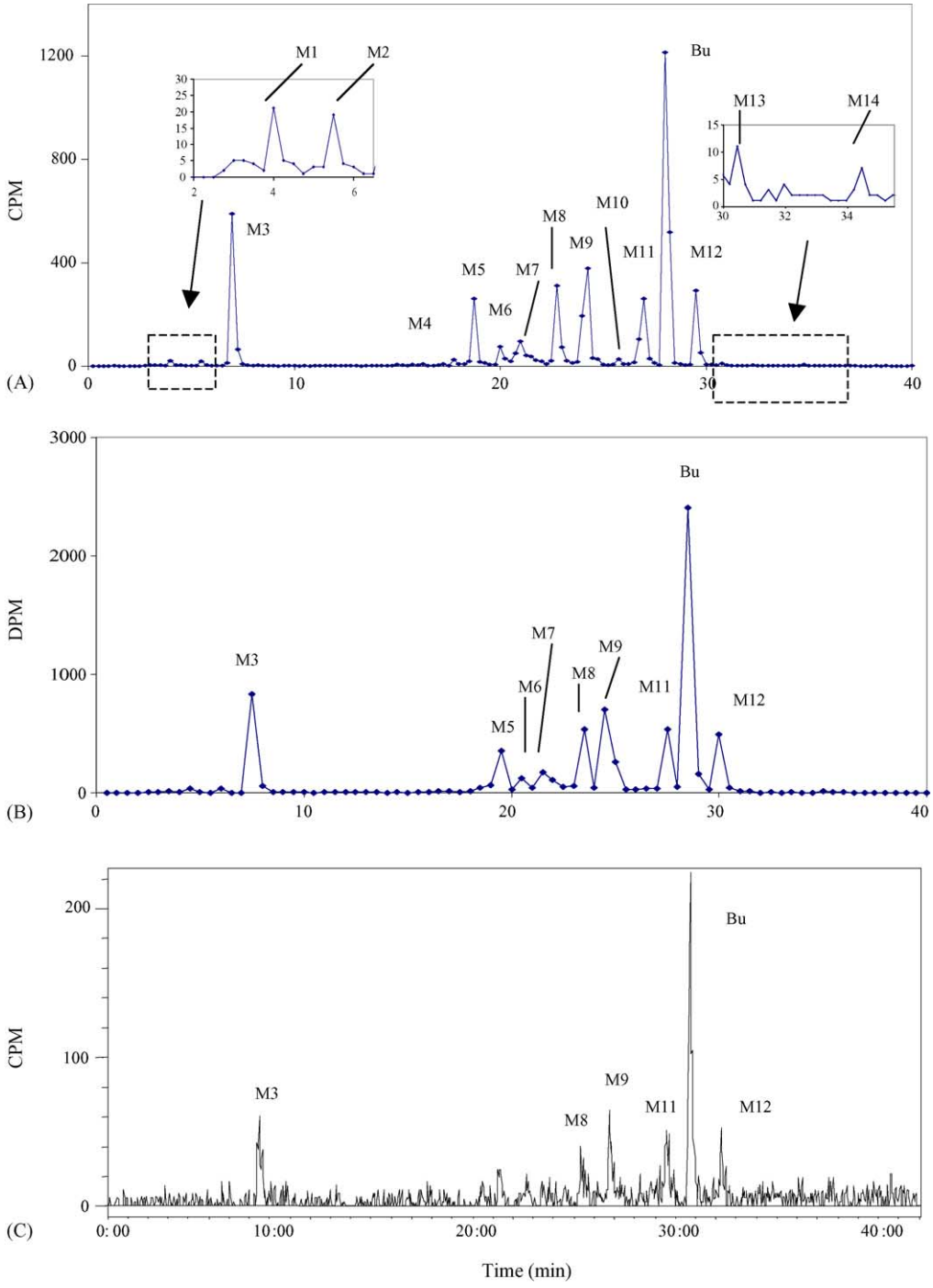


Fig. 2. Metabolite profiles of buspirone in human liver microsomes determined by HPLC coupled with MSC, LSC and RFD. A human liver microsome incubation sample of [^{14}C]buspirone was injected into HPLC (System I). Radioactivity in HPLC eluent was determined by (A) topcount: 8000 DPM injected (standard procedure); (B) LSC: 8000 DPM injected, two fractions per min, 10 min counting time; and (C) RFD: 32,000 DPM injected. The structures and naming system of major buspirone metabolites, M3 (1-PP), M5 (3′-OH Bu), M8 (Oxa-Bu), M9 (6′-OH-Bu), M11 (5-OH-Bu) and M12 (Bu *N*-oxide), were previously described [24].

better sensitivity (Table 1). An increase of counting time can improve the counting sensitivity. Usually, a radioactive peak is counted for only 5–15 s in RFD analysis, while MSC and LSC count each fraction for 10 min or more time. This is the reason why MSC and LSC have much better radiodetection sensitivity than RFD.

3.2. Precision and accuracy

Comparison of reproducibility for metabolite profiling by the three liquid radiochromatographic techniques is summarized in Table 3. The analytical precision of metabolite profiling by HPLC-TopCount ranged from 2 to 11%, which were

Table 2
Precision of TopCount for quantitative analysis of low levels of radioactive metabolites

Metabolite[a]	M1	M2	M4	M10	M13	M14
Radioactivity[b] (CPM)	32	30	32	42	23	15
R.S.D.[c]	14.6	10.8	14.6	5.3	9.0	27.8

[a] Buspirone metabolites in HLM were profiled by HPLC with TopCount (see Fig. 2A).
[b] The mean of metabolite radioactivity ($n = 5$), which was calculated based on five injections.
[c] R.S.D. is relative standard deviation.

comparable to those determined by the LSC and RFD methods (Table 3). TopCount provided a good measurement (relative standard deviation < 15%) for low level metabolites, M1, M2, M4, M10 and M13 (23–42 CPM, Table 2). The relative standard deviation for a trace metabolite (M14, 15 CPM) was 28% (Table 2). The accuracy values for the determination of buspirone metabolite profile (%relative radioactivity abundance) by HPLC-TopCount were ±2–12% (^{14}C-isotope, 10 min counting time), comparable to those determined by HPLC–RFD (Table 4).

3.3. Matrix effects

Matrix effects of human plasma, urine, fecal homogenates and liver microsomal incubations on TopCount performance were evaluated. Fig. 3 displays representative radiochromatograms with and without the injection of biological matrixes. Infused radioactivity without injection of a biological sample was within the range of ±15% from the mean values for both TopCount (Fig. 3A) and LSC (Fig. 3B).

Table 3
Precision of metabolite profiling by LSC, MSC and RFD

Radioactivity peak	Percentage radioactivity[a]					
	LSC		RFD		MSC	
	Mean[b]	R.S.D.[c]	Mean	R.S.D.	Mean	R.S.D.
M3	11.24	7.0	11.73	8.8	12.56	4.5
M5	5.96	10.8	5.40	12.7	6.00	3.1
M6	6.88[d]	10.0[d]	2.17	11.5	2.18	10.5
M7			4.77	11.4	4.92	4.9
M8	8.22	8.4	8.05	8.6	7.68	3.4
M9	13.20	5.0	13.42	3.5	12.92	4.2
M11	7.98	4.6	7.89	10.4	7.90	1.6
Buspirone	32.80	4.3	34.96	7.5	33.00	2.9
M12	7.26	3.7	6.84	8.7	6.60	2.1

[a] Profiles of buspirone metabolites were determined by HPLC with three radiodetection techniques (see Fig. 2). Percent distribution of radioactive peaks was calculated by dividing the radioactivity of a metabolite peak by the total radioactivity determined in the HPLC run (~8000 DPM per injection for HPLC–SLC and HPLC–MSC, and ~32,000 DPM per an injection for HPLC-RCD).
[b] Mean of the percentage radioactivity ($n = 5$), which was calculated based on five HPLC injections.
[c] R.S.D. is the relative standard deviation.
[d] The value represents the total of M6 and M7 radioactivity since the two metabolites were not separated in HPLC–LSC analysis.

Table 4
Accuracy of metabolite profiling by HPLC–MSC and HPLC–RFD[a]

Radioactivity peak	Percentage accuracy of radioactivity distribution[b]	
	TopCount	RFD
M3	+11.7	+4.3
M5	+0.7	−9.4
M6 and M7	+3.2	+0.9
M8	−6.6	−2.1
M9	−2.1	+1.7
M11	+1.0	−1.1
M12	−0.6	+6.6
M13	+9.1	−5.8

[a] Profiles of buspirone metabolites were determined by HPLC with three radiodetection techniques (see Fig. 2). The mean ($n = 5$) of percentage radioactivity distribution of each metabolite was calculated by dividing the radioactivity of the metabolite peak by the total radioactivity determined in the respected HPLC run.
[b] Percentage accuracy of radioactivity distribution was calculated using the percentage radioactivity distribution ($n = 5$) determined by LSC as "true values".

Extracts or concentrates of human samples equivalent to 1 mL plasma (Fig. 3D), 2 mL urine (Fig. 3E), or 1 mL liver microsomal incubation (Fig. 3C) had no or insignificant matrix effects except for the reduction of CPM values at the retention times corresponding to the HPLC solvent front in the chromatograms of the liver microsome and urine samples. Unlike the plasma sample, the urine and HLM samples were not treated with solid phase extraction. Most likely, significant amounts of proteins, salts and other polar components in these samples were eluted in the solvent front and deposited on the bottom of wells after solvent evaporation. These components might generate chemical quenching or act as a shield between radiolabels and solid scintillators, resulting in a lower counting efficiency. The same phenomenon was observed in MicroBeta counter analysis after 100 μL rat urine was injected [22]. Lower CPM values were also observed in radiochromatograms of the fecal extract (at fractions 84–88, Fig. 3F). This might have been caused by the same chemical quenching or shielding effect by endogenous components that eluted at this time rather than color quenching because these fractions did not display an intense color. Compared to MicroBeta counter [22], the matrix effects of urine and fecal samples on TopCount performance appeared to be minimal, suggesting TopCount is better for analyzing these samples.

The quenching observed in the fecal sample analysis was proportional to the amount of fecal extract injected (data was shown). Therefore, it could be minimized to an accepted level by limiting the amount of sample injected. Based on the data from this study (^{14}C isotope) we recommend that injection of human samples (equivalent to original volume or mass) should be ≤1 mL for liver microsomal incubations and plasma, ≤2 mL for urine, and ≤50 mg for feces. In addition, elution of polar metabolites in the HPLC solvent front should be avoided. Since feces displayed the most significant matrix effect among the samples tested,

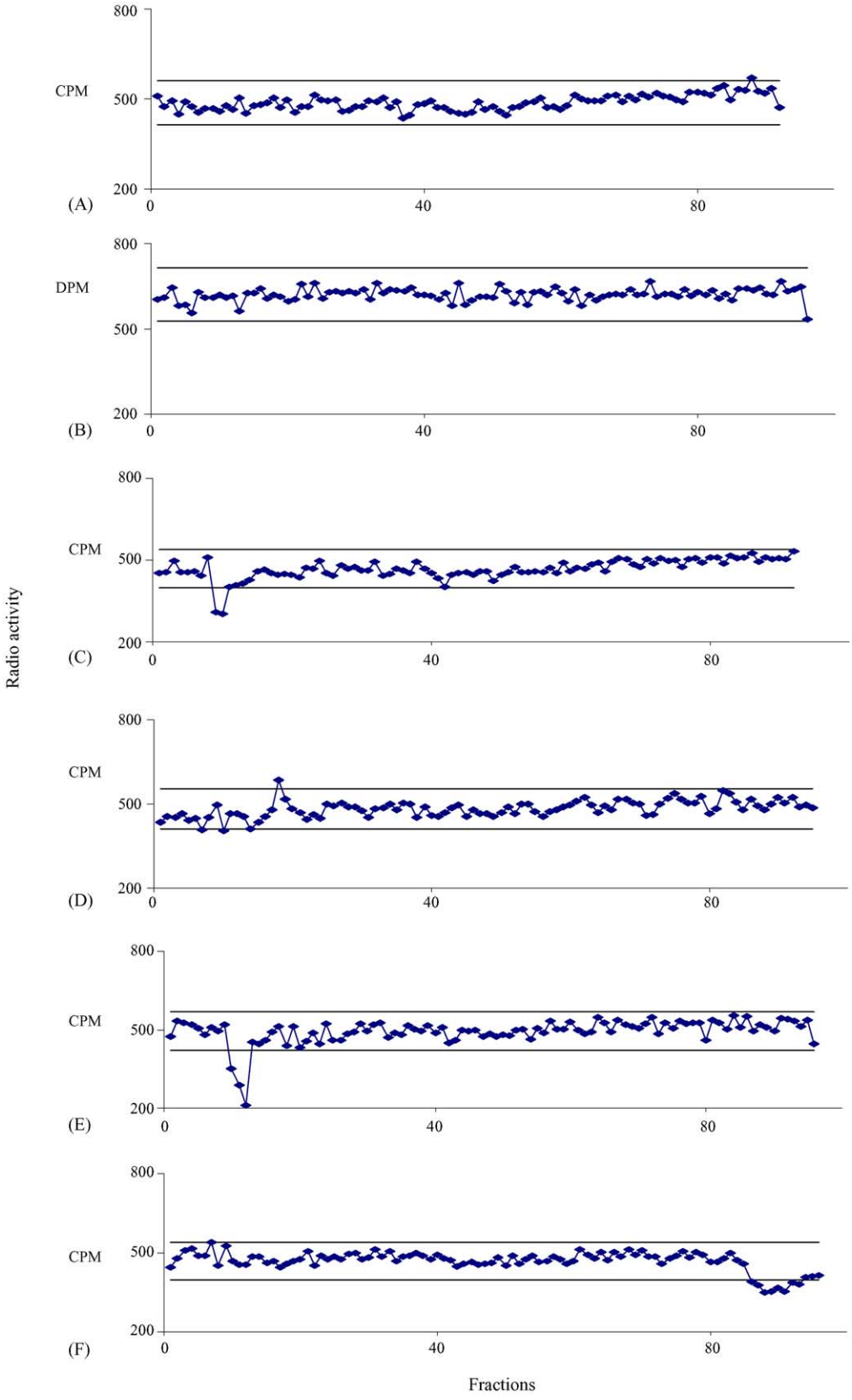


Fig. 3. Matrix effect of human plasma, urine, feces and liver microsomal incubations. Extracted or concentrated biological samples were injected into HPLC (System II) followed radioactivity analysis by TopCount (standard procedure). (A) TopCount control, no injection of a biological sample; (B) LSC control, no injection of a biological sample; (C) HLM (equivalent to 1 mL); (D) human plasma equivalent to 1 mL); (E) human urine (equivalent to 2 mL); (F) human fecal homogenate (equivalent to 100 mg feces). The up and down lines in each figure represent ±15% values from mean.

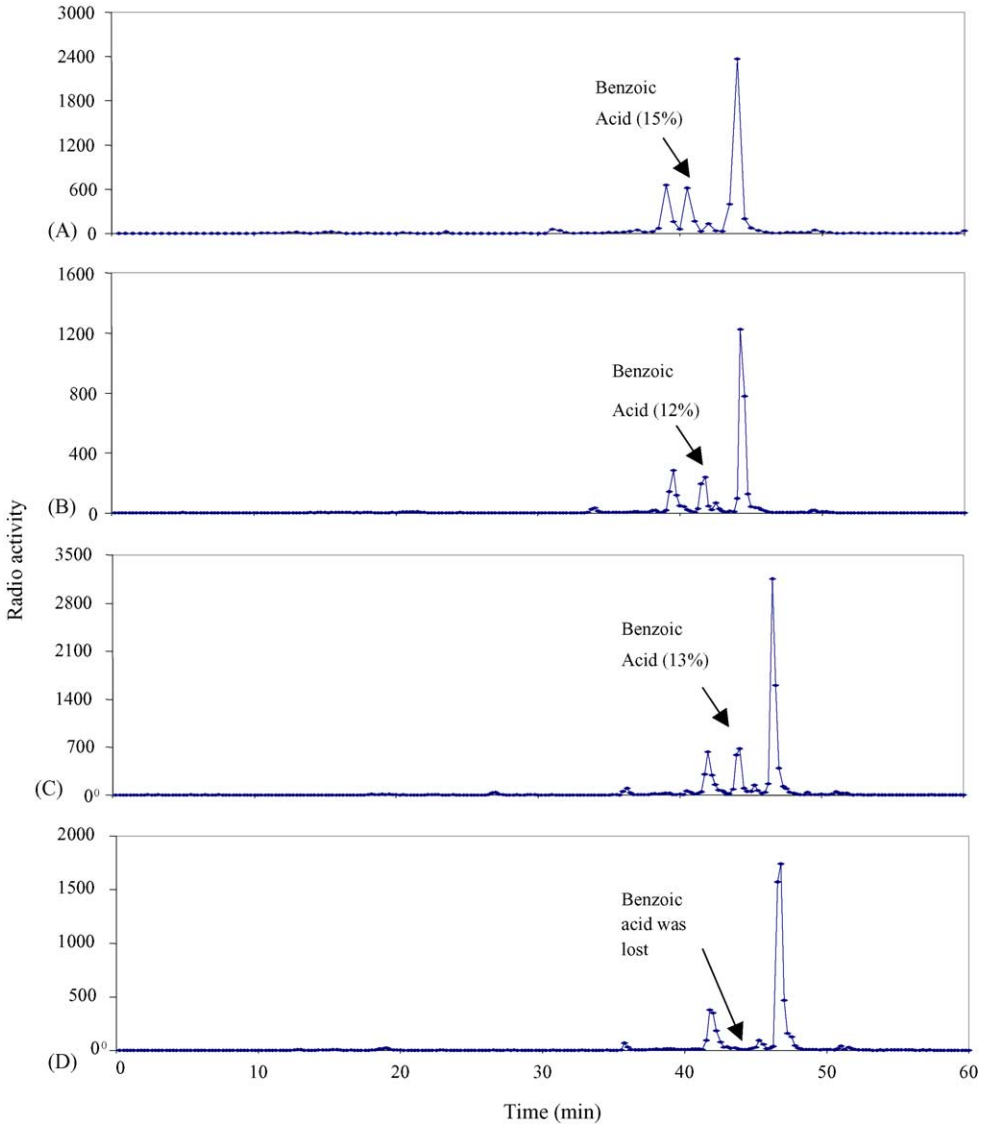


Fig. 4. Analysis of volatile metabolite using various HPLC–MSC techniques. A incubation mixture containing [^{14}C]benzoic acid, a volatile metabolite, was profiled by HPLC (System IV) with radiodetection (MSC or LSC). (A) Metabolite profile determined by HPLC–LSC; (B) Metabolite profile determined by HPLC-TopCount after drying the microplates; (C) Metabolite profile determined by HPLC–MicroBeta counting after direct addition of cocktail into microplates; (D) Metabolite profile determined by HPLC–MicroBeta counting after drying the microplates.

it is highly recommended to inject as little as possible fecal samples. Furthermore, when dealing with radioisotopes other than the ^{14}C isotope or the biological matrices different from those tested in this study, potential matrix effects of the samples on TopCount performance should be evaluated.

3.4. Volatile metabolite analysis

The standard HPLC–MSC method requires solvent evaporation, which is usually carried out using a speed vacuum device. To determine if volatile metabolites are evaporated during the drying process, an HLM incubation sample containing a volatile metabolite, benzoic acid, was analyzed using both TopCount and MicroBeta counter. The radiochromatograms determined by HPLC–LSC (Fig. 4A) and HPLC-TopCount (Fig. 4B) methods were very similar, indicating benzoic acid was not evaporated in the TopCount analysis. The observations suggest that volatile metabolites may retain on LumaPlates® during the drying process. Most likely, radioactive metabolites tightly attached to yttrium silicate particles. For the same reason, an attempt to recover metabolites from dried LumaPlates® failed (data not shown). However, benzoic acid was completely lost in the analysis by MicroBeta counter (Fig. 4D). The loss of the volatile metabolite was avoided when liquid scintillation cocktail was directly added to Scintiplate without solvent evaporation (Fig. 4C). The observations suggest that TopCount

Table 5
Radioactivity recovery determination in metabolite profiling by HPLC–MSC

DPM[a]	2458 ± 125[e]
DPM_C[b]	2360 ± 90
DPM_M[c]	2484 ± 57
DPM_T[d]	2341 ± 76
Column recovery (%)[e]	96
Microplate recovery (%)	101
Total recovery (%)	95

[a] DPM is the radioactivity of the HPLC eluent without passing through HPLC column and no solvent evaporation.
[b] DPM_C is the radioactivity in the HPLC eluent passing through HPLC column without solvent evaporation.
[c] DPM_M is the radioactivity of the HPLC eluent without passing through HPLC column and with solvent evaporation.
[d] DPM_T is the radioactivity of the HPLC eluent passing through HPLC column and with solvent evaporation. Recoveries were calculated based on Eqs. (5)–(7).
[e] Mean value ± S.D. ($n = 4$).

may be able to retain some volatile metabolites, which depends on volatility and p*K* values of metabolites and pH values of HPLC solvents. Therefore, to ensue analytical quality of HPLC–MSC analysis it is necessary to determine microplate radioactivity recovery in addition to column recovery.

3.5. Radioactivity recovery determination

An LSC-based method was developed for determining radioactivity recovery in HPLC–MSC analysis (Section 2.4.4). By using this method, column, microplate and total radioactivity recoveries of metabolite profiling of a rat urine sample were determined (Table 5), which confirmed that no significant amounts of radioactivity retained on the HPLC column or lost in the solvent evaporation. This example suggests that radioactivity recovery from HPLC–MSC analysis can be rapidly and reliably determined. If the total radioactivity recovery of an HPLC–MSC method is good it would not be necessary to calculate HPLC column and microplate recoveries.

3.6. Method application

3.6.1. Profiling of low levels of radioactive metabolites in plasma

One of the major applications of HPLC–MSC in drug metabolism is to detect and profile in vivo metabolites, especially plasma metabolites. Usually, the amounts of plasma samples available for analysis are very limited, and metabolite concentrations in plasma are relatively low compared to those in urine, feces and bile samples. Fig. 5 is a typical plasma radiochromatogram determined using HPLC-TopCount. A minor metabolite (7 CPM) at retention time 34 min was clearly detected, consistent with the calculated limit of detection for TopCount (5 DPM, Table 1). Based on the sensitivity of TopCount, we recommend injecting more than 500 DPM of radioactivity for each analysis (10 min counting time) of ^{14}C labeled metabolites. Therefore, a minor metabolite (10 DPM) corresponding to 2% of the total injected radioactivity can be detected. For quantitative analysis of the same metabolite by TopCount, approximately 1500 DPM of radioactivity may be required. Alternatively, increasing MSC counting time to 20–30 min could significantly improve its sensitivity.

3.6.2. Analysis of in vitro phase II metabolites using radiolabeled co-factors

Another application of HPLC–MSC in drug metabolism is the analysis of phase II metabolites using radiolabeled

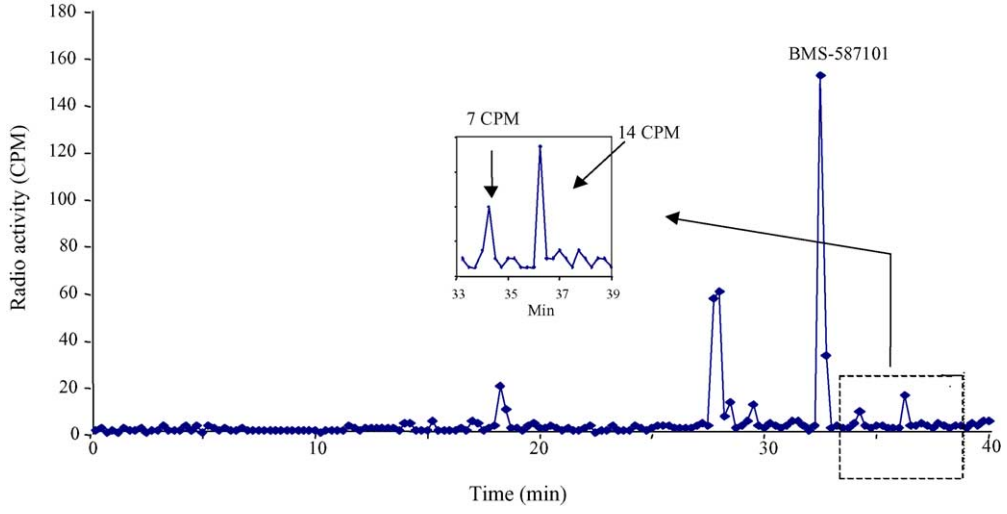


Fig. 5. Analysis of low level radioactive metabolites of BMS-214778 in rat plasma by HPLC-TopCount. A plasma extract of rats after the administration of [^{14}C]BMS-214778 was analyzed by HPLC (System III) with TopCount (standard procedure). Approximately 493 DPM radioactivity was injected.

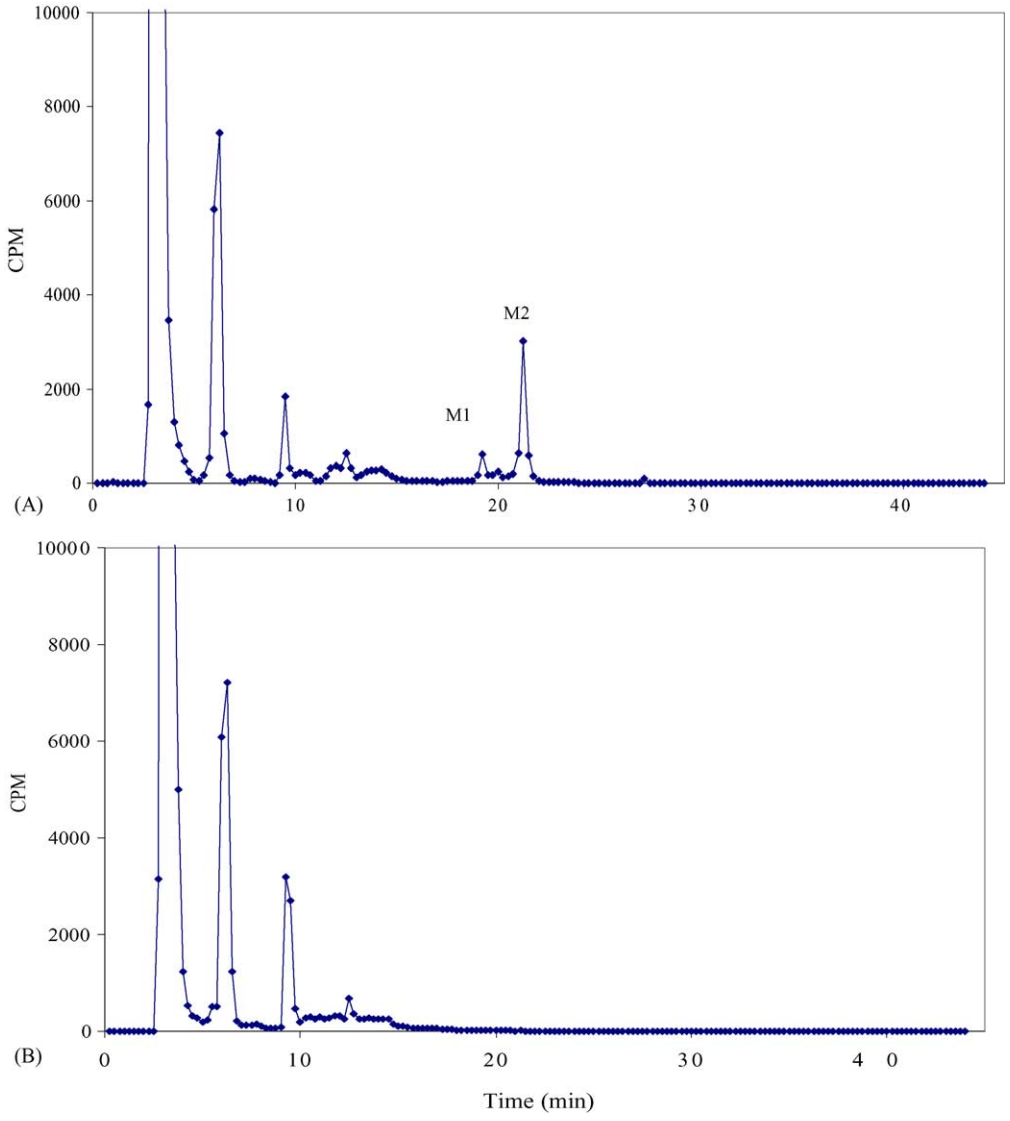


Fig. 6. Analysis of [^{3}H]GSH trapped reactive metabolites by HPLC with topcount. A nonlabeled drug (50 μM) was incubated with a mixture of GSH (1 mM) and trace [^{3}H]GSH (1–2 μCi/mL) in human liver microsomes. After precipitating proteins, the samples were analyzed by HPLC (System V) with TopCount (standard procedure). (A) Radioactivity profile of the incubation with NADPH. M1 and M2 were GSH-trapped reactive metabolites; (B) radioactivity profile of a control incubation without NADPH.

co-factors such as glutathione (GSH). Fig. 6 presents radioactivity profiles of a non-radiolabeled drug in human liver microsomal incubations in the presence of 1 mM [^{3}H]GSH with or without the addition of NADPH. GSH-trapped reactive metabolites M1 and M2 were detected by HPLC–MSC (Fig. 6A), but not seen in the incubation sample without NADPH (Fig. 6B). The results indicate that cytochrome P450 catalyzed the formation of reactive metabolites that were trapped by GSH. Usually, formation of reactive metabolites represents a minor metabolic pathway. The MSC technique enables to detect low levels of GSH adducts even with a mixture of a small portion of [^{3}H]GSH and non-labeled GSH. To analyze the same level of the GSH adducts by RFD, 50–100-fold more [^{3}H]GSH would be needed in the incubations.

3.6.3. Determination of metabolite concentrations in incubations

To illustrate the utility of MSC for quantitative analysis of low levels of radioactive metabolites in enzyme kinetic studies, [^{14}C]buspirone was incubated with human liver microsomes at a low substrate concentration (0.5 μM), and microsomal protein concentration (0.2 mg/mL). Fig. 7 is a typical profile of buspirone metabolites (40–110 CPM per peak) formed at low levels of the substrate concentration. The concentrations (0.032–0.101 μM) of buspirone metabolites in the incubation medium were determined

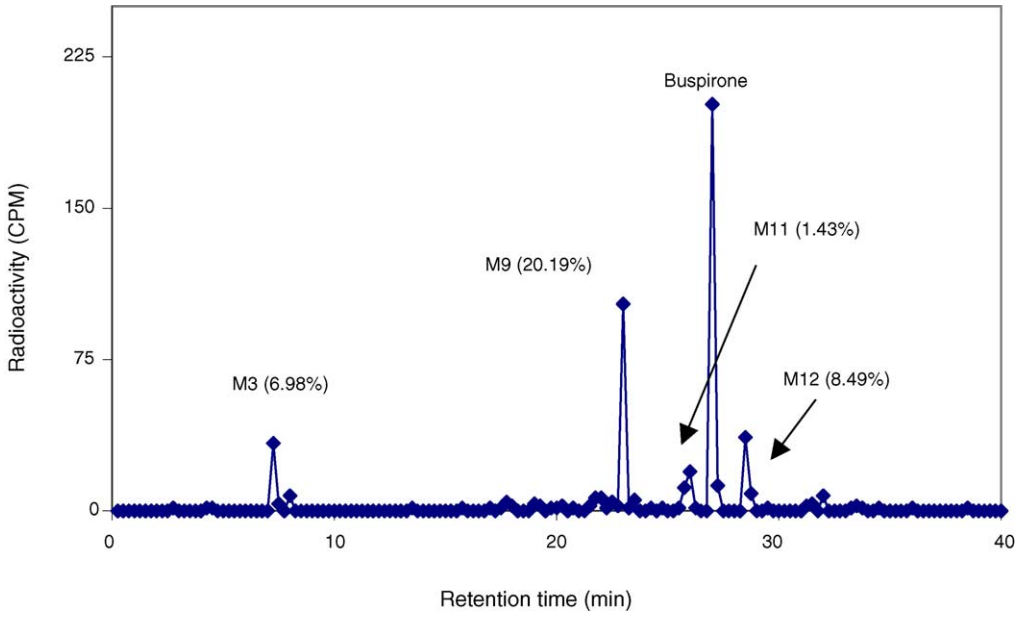


Fig. 7. Determination of metabolite concentrations of [^{14}C]buspirone in human liver microsomes by HPLC with TopCount. Buspirone (0.5 μM) was incubated with HLM (0.2 mg protein/mL) for 5 min. Metabolite profiling (%distribution of radioactivity) was obtained using HPLC (System I) with TopCount (standard procedure) after 200 μL supernatant was directly injected. Metabolite concentrations were determined based on the percent distribution of radioactivity (Section 2.5.3). Major buspirone metabolites formed at the low substrate concentration in HLM were M3 (1-PP, 0.035 μM), M9 (6′-OH-Bu, 0.101 μM), M11 (5-OH-Bu, 0.032 μM) and M12 (Bu *N*-oxide, 0.042 μM). The structures of these metabolites were previously described [24].

based relative radioactivity abundance of metabolite peaks (see Section 2.5.3.).

4. Conclusions

Although MSC as an off-line liquid radiochromatographic detector has been available only for the past few years, it has shown a great promise as an alternative tool for HPLC profiling of radioactive metabolites. For metabolite detection and quantification, TopCount was 50–100-fold more sensitive than RFD, and approximately two-fold more sensitive than LSC. For determining relative abundance of metabolites, TopCount had comparable accuracy and precision to RFD, and comparable precision to LSC. Human samples such as liver microsomal incubations, plasma and urine had no or minimal matrix effects on the analysis of ^{14}C isotopes by TopCount. However, extracts from more than 50 mg human feces resulted in significant quenching. TopCount was able to detect benzoic acid, a volatile metabolite that was completely lost in the analysis by MicroBeta counter. Radioactivity recovery in HPLC–MSC analysis, including column, microplate and total recovery, was reliably determined using an LSC-based method. Applications of HPLC–MSC techniques to the profiling of minor metabolites in plasma, analysis of [^{3}H]GSH-trapped reactive metabolites, and determination of metabolite concentrations in an enzyme kinetic experiment demonstrate that the HPLC–MSC technique is especially useful for detection and quantitative determination of low levels of radioactive metabolites in biological matrices.

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