

Application Note

TopCount

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Using the TopCount® Microplate Scintillation and Luminescence Counter and Deep-Well LumaPlate™ Microplates in Combination with Micro Separation Techniques for Metabolic Studies

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Abstract

For biotransformation investigations of *in vivo* and *in vitro* studies, radioactively labeled drugs provide one of the most important tools to completely track the metabolites in complex biologic matrices, such as blood, urine, feces, bile, and *in vitro* samples. Mass spectrometry is another important method for tracking metabolites with or without the use of stable isotopes. Most metabolites can be detected with this technique. However, without calibration curves for all metabolites, quantitative results are difficult to obtain with mass spectrometry because peak intensities of LC/MS (Liquid Chromatography/Mass Spectroscopy) strongly depend on the ionization efficiency of the individual metabolites. In contrast to the MS approach, radioactively-labeled metabolites will always have the same response on a radioactivity detection system. Therefore, peak intensities of such measurements are directly related to concentrations. In most of these studies, ¹⁴C or ³H is used for radiolabeling of a given drug. From the analytical point of view, the radioactivity should be as high as possible to detect the weak signal intensities of metabolites (e.g., in dilute excreta collected several days after dose administration). However, for various reasons the amount of radioactivity should be reduced in all applications; therefore, methods are needed to reduce the radioactivity to the lowest amount that will provide adequate sensitivity. Moreover, the time pressure to perform studies faster is an issue in every pharmaceutical company. With the TopCount® microplate scintillation and luminescence counter from Packard Instrument Company, it is now possible to perform faster analytical investigations using much less radioactivity. Another advantage of this technology is the possibility of using the TopCount system as a highly sensitive off-line radioactivity detector for nano- and micro-separation techniques, such as HPLC (high per-

formance liquid chromatography), CEC (capillary electrochromatography) and CE (capillary electrophoresis). Because of the very small peak volumes and the small amount of radioactivity, on-line detection with a conventional radiomonitor is not sensitive enough. It is also not possible to measure peaks with half-widths of a few seconds.

Introduction

When combined with μ -HPLC (micro-High Pressure Liquid Chromatography), the TopCount microplate scintillation and luminescence counter can dramatically improve the metabolism laboratory work. The HPLC system performs the chemical separation, and the samples are automatically dispensed into special, opaque 96 deep-well microplates, called Deep-Well LumaPlate™ microplates (Packard Instrument Company, Meriden, CT, U.S.A.), using a fraction collector. These special microplates contain a solid scintillator, which is on the bottom of each well. Samples that are collected in the Deep-Well LumaPlate microplates are dried using a vacuum centrifuge. The TopCount system will count these plates quickly (up to 12 samples at a time) and accurately. For μ -HPLC applications, much smaller samples are required, which greatly reduces the consumption of solvents, and at the same time enhances the sensitivity. For example, the reduction of the column ID (internal diameter) from 4.6 mm (1 mL/min flow) to 2.0 mm ID (0.2 mL/min flow) improves the sensitivity by a factor of five, and with a 0.5 mm ID the factor would be 100. Because of the higher peak concentrations in μ -HPLC applications, and the low flow rate, the TopCount approach fits perfectly. With a μ -HPLC flow rate and a short fraction collection time, the Deep-Well plates will be filled only with a small amount



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of solvent, which for these experiments was 50 microliters. This small sample volume facilitates a short drying time and high peak intensities. Ideally, this would most often be represented as one peak in one well for the highest sensitivity. This means that peak widths should be smaller than the fraction collection time. However, this is only possible in high resolution separation techniques, such as μ -HPLC or CE.

Materials and Methods

For the HPLC separation of rat urine, a 2.1 mm ID column with 3 μ m particle size and a length of 150 mm (X-Terra, Waters Corp., U.S.A.) on an HPLC instrument (HP1100 Hewlett-Packard, Waldbronn, Germany) were used. For sample cleanup of the biological matrices, a second column (4.0 x 40 mm, BioTrap 500MS, Chromtech, Hagersten, Sweden) filled with RAM (Restricted Access Material) was connected to the system in the back flush configuration. A second six-port valve was connected to either the analytical column or the BioTrap with the radioactivity detection system. With this configuration, it became possible to detect the amount of radioactivity that could not be trapped, which is very important for quantitative aspects of a metabo-

lism study. The complete instrumental setup is shown in Figure 1.

Typically, five to 300 μ L of blood, serum, plasma or urine, without further sample pretreatment, were injected. Water, with 20 mM ammonium acetate adjusted to pH 9, was used as the extraction solvent for basic compounds. After the drug and its metabolites were trapped on the BioTrap column, which is essentially an SPE (Solid Phase Extraction) column, the column valve was switched and the compounds were separated using the analytical column under gradient conditions. Solvent A of the gradient was comprised of acetonitrile with 10 mM triethylamine, and solvent B was comprised of water with 50 mM ammonium acetate and 10 mM triethylamine. All solvents and chemicals were purchased from Fluka (Buchs, Switzerland). After passing through the UV-detector, the HPLC fractions were collected into Deep-Well LumaPlate microplates using a fraction collector (FC 204, Gilson Inc., Middleton, WI, U.S.A.) with a collection time of 15 seconds per well and 10 seconds for the 0.5 mm ID column. The LumaPlates, containing 25 - 50 μ L per well, were dried on a SpeedVac[®] (SuperADN, Savant Instruments Inc., Holbrook, New York, U.S.A.) for 35 minutes at 43°C. Samples from the small column could be dried in only 5 minutes, because

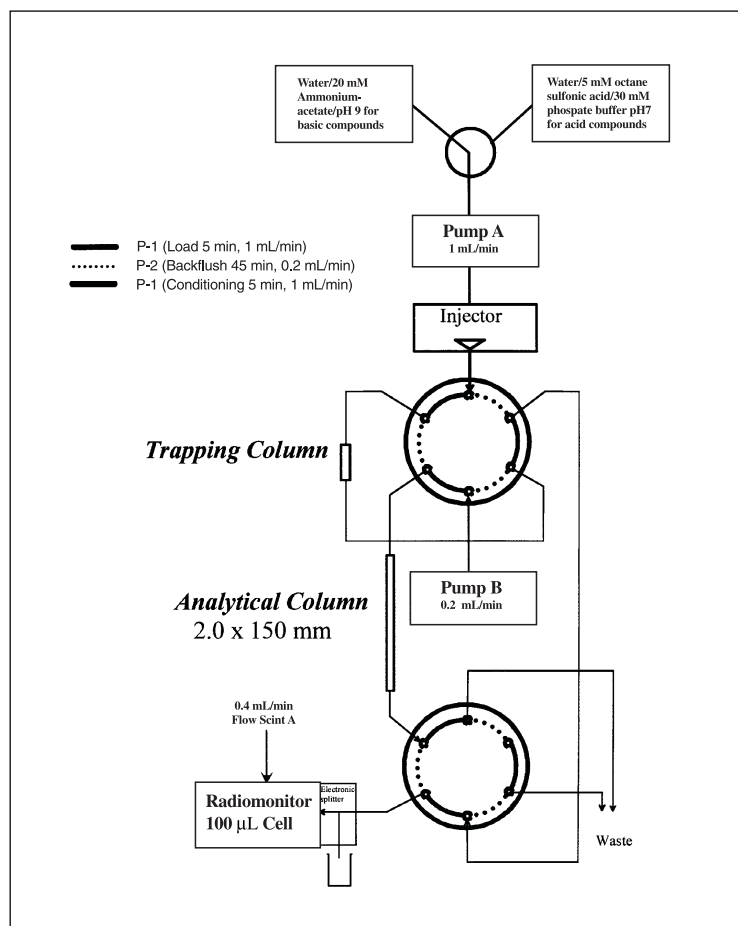


Figure 1.

Setup of the HPLC instrumentation with the radiomonitor detector.

each well contained only 2 microliters of sample eluate and 25 microliters of ethanol from the sheath flow. They were then sealed and placed in the TopCount system for counting. Prior to counting, the TopCount system was calibrated, the 12 detectors were normalized, and the backgrounds of the photomultipliers were determined. Then the microplates were counted, using the 12 simultaneous counting detectors, for eight minutes per sample. After less than four hours of sample counting, the counting results of the samples for the 60-minute HPLC run were stored as ASCII files. The ASCII files were converted for integration and analysis using ASCII FLO conversion software, and then analyzed using the FLO-ONE™ analysis software (Packard Instrument Company). This provided results in a manner similar to that which is performed on-line using the Packard Radiomatic™ model 505TR flow scintillation analyzer, which is an on-line detector for HPLC applications.

For on-line radioactivity monitoring of the same samples, a radioactivity flow detector (Radiomatic™ 505TR, Packard Instrument Company, Meriden, CT, U.S.A.) was operated with a 100 μL detection cell. The liquid scintillator was Flo-Scint™ A (Packard Instrument Company, Meriden, CT, U.S.A.), and a flow rate of 800 $\mu\text{L}/\text{min}$ was used.

For the separation with the 0.5 mm ID column a 12 $\mu\text{L}/\text{min}$ flow was generated with the Acurate™ flow splitter from LC Packings (Amsterdam, The Netherlands). The column itself was 15 cm long, filled with Luna C18, 3 μm material and purchased from Phenomenex (Torrance, California, U.S.A.). Because of the low flow rate, the fraction collector was modified with a sheath liquid adaptor as shown in Figure 2. With sheath flow of 150 to 300 $\mu\text{L}/\text{min}$, a drop rate of 24 to 45 drops/min could be

achieved. A security guard column 2x4 mm (Phenomenex) filled with a 2 mm cartridge of C18 material was used as a trap. The analytical column was connected directly to the fraction collector with 75 μm ID fused silica capillary.

Results

The chromatogram in Figure 3 shows the result of two HPLC runs of the same rat urine. Curve B represents a 100 μL injection with 61,500 DPM of ^3H labeled drug and its metabolites measured on-line using the Packard Radiomatic Model 505TR flow scintillation analyzer. Curve A represents a 7 μL injection with only 4,300 DPM of total radioactivity measured off-line in LumaPlate microplates with the TopCount system. For better visualization, the upper curve of A was also expanded by a factor of five. When comparing the classical on-line and the new off-line approaches, it becomes clear that curve A shows much more detail than does curve B. In addition to using a 14-fold smaller injection volume, the TopCount/LumaPlate combination provides better sensitivity because of a much lower background, while maintaining very high counting efficiency. On a blank LumaPlate microplate, the background was determined to be only 0-2 CPM. Every count above 4 CPM can be interpreted as a valid tritium signal. It should also be noted that all peak intensities in both curves (A and B) compare well to each other. The main metabolite at a retention time of 16.5 minutes is clearly visible in both curves (A and B). This peak measured by the TopCount shows a better chromatographic resolution than the on-line chromatogram. In addition, all peaks, including the very small ones at retention times of 11, 15, 28, 31 and 40 minutes, were found in other samples of the *in vivo*

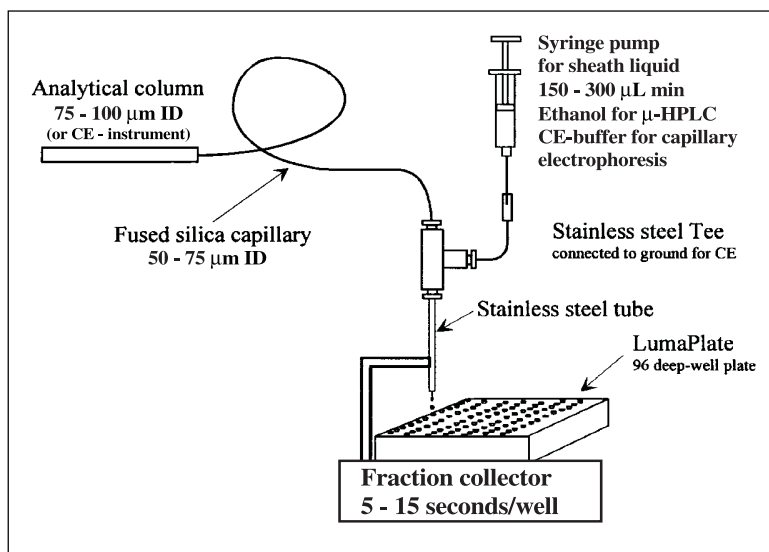


Figure 2.

Experimental setup of the sheath liquid option for the fraction collector.

study, such as in samples collected at different time points after drug administration, in plasma and in some feces samples. Additionally, the broad trace of radioactivity from 10 to 58 minutes was reproducible with these other samples.

When compared to a classical LSC (liquid scintillation counter), the throughput of the TopCount/LumaPlate combination is much greater; less than four hours of counting time vs 30 hours of counting time for LSC (240 samples x 3 x 2 minutes per sample) to achieve a similar result. In addition, the LSC background is typically 12-16 CPM vs the 0-2 CPM for the TopCount. This means that the small peaks at the retention time of 11, 31.5 and 40 minutes would not be visible in a classical LSC. It should also be noted that the TopCount system is even more sensitive for ^{14}C labeled compounds than for ^3H , because of higher counting efficiency for this radioisotope. See Table 1.

For a determination of the reproducibility of the TopCount, the LumaPlate microplates of an HPLC run were measured twice. Figure 4 shows the results of two individual Topcount measurements of the same LumaPlate. For the rat chromatogram, 250 μL of rat plasma were injected. The sample was taken at a late time point in the study, when the drug was almost com-

pletely excreted. In general, samples such as this have a very small amount of total radioactivity, which is often distributed over a larger number of metabolites. Therefore it is very difficult to collect enough material for an analysis. The total radioactivity of this injection was previously determined to be 1,722 DPM. In this case, the TopCount system could reproduce the data with a high degree of accuracy, especially for the very low amounts of radioactivity. Also, similar results can be achieved by the reproducibility of the complete HPLC run, including the drying step. (Data not shown.)

The TopCount system's technology offers the possibility to easily measure radioactive samples which was not previously practical. In nano and micro separation techniques it was not possible to measure it at all. For example, measurement of a 20 CPM peak and with a peak width of a few seconds moving in a fused silica capillary was not even possible. However, using a fraction collector with a sheath flow option and the LumaPate microplates, makes the difference. With fraction collection times between 3 and 20 seconds, using 15 and 30 μL per well, the drying time on the SpeedVac is reduced to 5 minutes. As it is shown in Figure 5, a high quality radio chromatogram can be achieved from a narrow-bore HPLC system. The high peak concentrations, which can be achieved with these nano and microcolumns, offer higher sensitivity for mass spectrometers. For example,

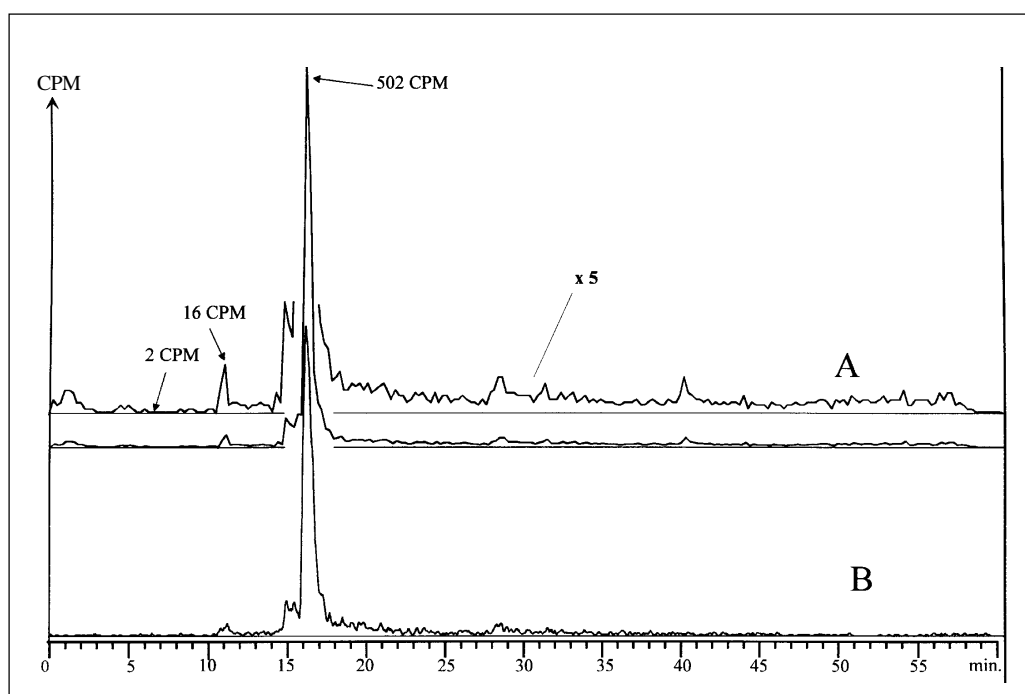


Figure 3.

Radioactivity chromatogram of rat urine containing ^3H labeled drug.

A = 4300 DPM (7 μL) injected and counted off-line with the TopCount system (8 minutes per well).

B = 61500 DPM (100 μL) injected and monitored on-line with adding 800 μL LSC-cocktail to a 100 μL mixing cell.

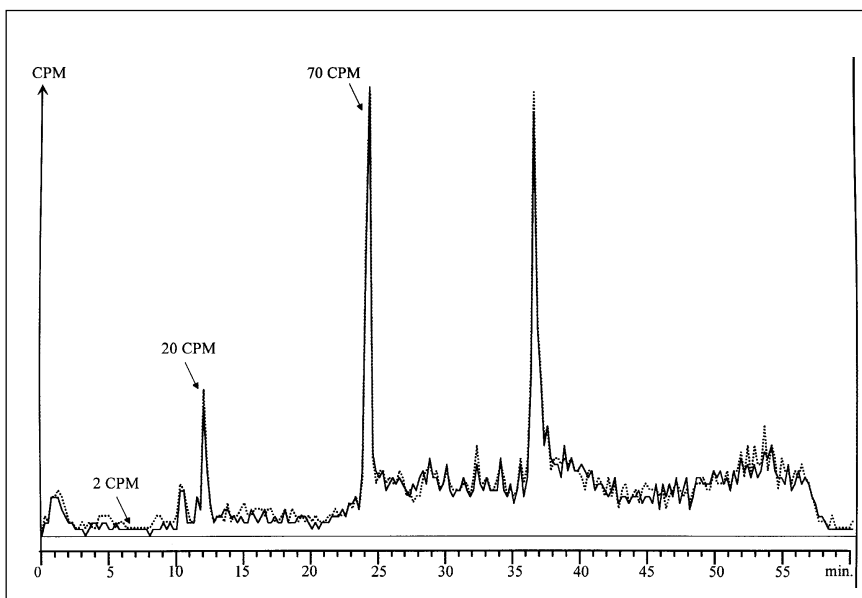


Figure 4.

Reproducibility of TopCount counting.
The two curves show a chromatogram of 1,722 DPM rat plasma containing ^3H labeled drug and its metabolites (250 μL injected).

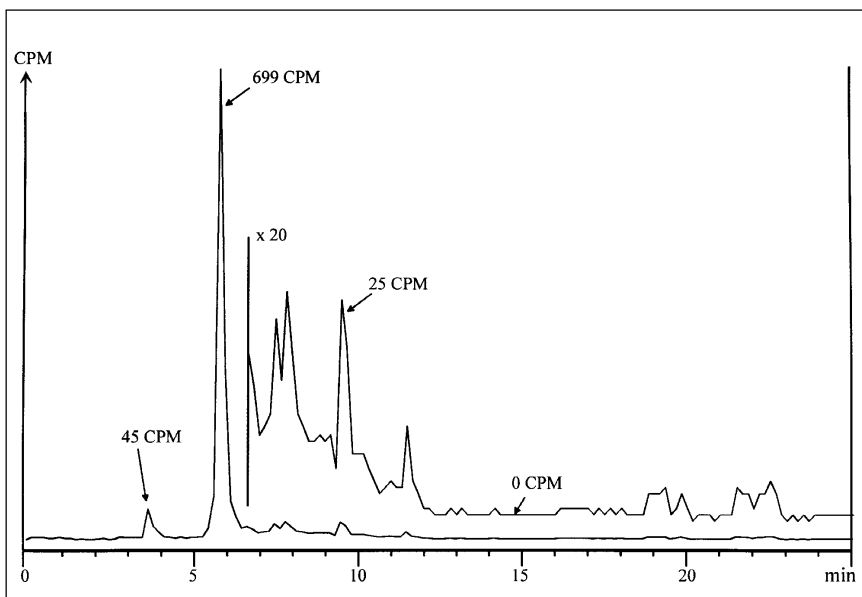


Figure 5.

Radioactivity chromatogram of rat urine containing ^3H labeled drug with 5500 DPM (5 μL) injected and counted off-line with the TopCount (8 minutes per well). The HPLC flow rate was 12 $\mu\text{L}/\text{min}$ on a 0.5 mm ID column with a length of 150 mm and filled with 3 μm C18 material. Sheath liquid is ethanol at 150 $\mu\text{L}/\text{min}$.

*Note: This is a similar sample as was used in Figure 3, but with a different gradient.
The small peak a retention time of 9.5 minutes could be identified as the parent drug.*

	³ H	¹⁴ C	¹²⁵ I	⁵¹ Cr	³³ P	³² P
Average CPM	98806	120549	175379	3221	108683	230259
Reference DPM	245891	132633	344379	12616	124379	262816
Average Efficiency	40.2%	90.9%	50.9%	25.5%	87.4%	87.6%
Average Bkgrnd. CPM	1.3	2.3	60.7	1.3	1.7	9.7
Average Crosstalk	0.002%	0.001%	0.255%	0.038%	0.002%	0.120%

Table 1.

Typical counting efficiencies for the Deep-Well LumaPlate microplate in the TopCount system.

Note: The data for Table 1 were supplied by Packard Instrument Company.

with 500 μ m ID column, a given signal is 100 times higher than it would be after a separation on a 4.6 mm column. In the past when using this advantage, the traces of the radioactive metabolites were lost. Now it becomes possible, without investing much more time, to have both sensitivity and radioactivity in an off-line approach. This becomes important in connection with mass spectrometry.

Conclusions

The examples shown in this paper clearly demonstrate that the TopCount system and the Deep-Well LumaPlate microplates are very useful for drug metabolism studies using off-line counting of HPLC runs. This method eliminates the need for scintillation cocktail, and it greatly reduces the labor required to prepare a large number of samples, since the work is almost completely performed by the HPLC, the fraction collector and the TopCount. The only manual operation that is required is to cover each plate with a TopSeal™ sealing film (Packard Instrument Company, Meriden, CT, U.S.A.) before loading the plates on the TopCount. Another advantage to using the TopCount with LumaPlate microplates is that it counts 96-well plates at a high speed, while an LSC takes approximately 10 times longer to count the same number of samples using one vial per sample.

A few limitations should be mentioned as well. The direct counting of plasma, urine and feces samples could lead to incorrect results, because the color and small particles of the samples can produce strong color quenching. In such cases, it is advisable to use a color quench correction curve on the TopCount to correct the CPM for the color quenching effect. In these situations, it is best to use a solid phase extraction method or the BioTrap that was used in the experiments for this application note for the initial cleanup of the sample to reduce these quenching effects. In some cases, such strongly colored samples may be more successfully counted via a conventional LSC.

There is no chemical quenching effect when using a LumaPlate microplate, but strong coloration of the drug or the metabolites can also reduce the counting efficiency. However, a visual inspection of colored dry LumaPlate wells can reveal which results may be suppressed. Decolorizing with H₂O₂ inside the LumaPlate microplates might also be an option in some cases.

To further maintain the counting efficiency, the actual sample material must not be volatile. The samples in a LumaPlate must be completely dry before counting. Volatile samples will be lost in the drying process.

It should also be noted that some compounds may have a high affinity for the walls of the LumaPlate microplate. This is unlikely for most polar compounds, but it may occur. Accordingly, it is highly recommended that the samples be dried in centrifugal vacuum evaporators, rather than through normal evaporation processes. Centrifugal evaporators, such as the SpeedVac help to ensure maximum placement of the sample on the scintillator of the LumaPlate. Of course, in biotransformation studies, on-line monitors can also exhibit sticking problems.

Overall, the TopCount system, together with all types of HPLC separations including conventional HPLC, CEC and CE can improve the analysis of radioactivity chromatograms in drug metabolism studies. However, the highest benefit of the TopCount is its ability to easily measure the smallest fractions from capillary electrophoresis separations and as well larger fractions from nano and micro-HPLC applications without any change of instrumentation.

Acknowledgment

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