LC-^{14}C-MS/MS: Compatibility of Capillary Columns for HPLC-^{14}C-MS/MS.

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Abstract

Radioactivity detection of ^{14}C-labeled compounds is an inevitable tool in the analysis of samples from ADME studies. The striking advantage of radioactivity detection is the strict quantitative information regardless of biotransformations of the parent compound, provided that the ^{14}C-label is situated in a metabolically and chemically inert part of the molecule. Against these advantages stands the fact that radioactivity detection is rather insensitive compared to other detection modes like fluorescence or mass spectrometry.
As HPLC columns with small inner diameters provide higher sensitivity, better compatibility to mass spectrometry due to lower flow rates, and as an additional benefit smaller sample consumption, a combination of capillary HPLC columns with ^{14}C- and MS/MS detection would be an almost ideal set-up for the investigation of all kind of ^{14}C-labeled compounds. Further on, the combination with mass spectrometry is highly useful for allocation of metabolites in the ion chromatogram when using biological samples. In consequence, it was investigated how far ^{14}C-detection can be microfied and examples for Capillary-HPLC-^{14}C- and MS-detection are shown.

Introduction

Radioactivity detection in combination with HPLC is mostly used in combination with 4 or at least 2 mm columns while improvement in resolution and also S/N ratio of capillary LC is reported [1-3] So, the question arises, whether the same or at least a similar effect can be observed in capillary HPLC with ^{14}C-detection. The following investigations were made in order to get a better understanding of the factors influencing the ^{14}C-signal. The contribution of:

a) the particle size of the scintillation material,
b) the inner diameter of the HPLC column
c) the volume of the detection cell
d) flow velocity

on resolution and signal intensities was investigated. The optimized set-up was then finally applied for the investigation of the metabolite pattern from microsomal incubations of a suitable drug candidate.

Material and Methods

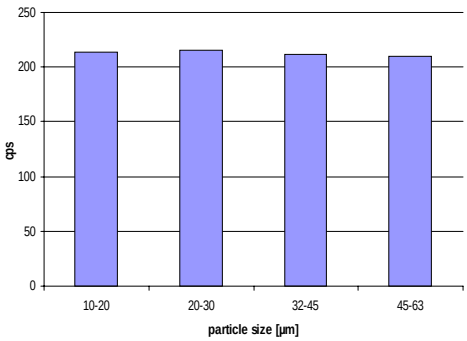
^{14}C-labeled compounds: Benzene, specific activity: 24.67 MBq/mg; Ethylbenzene, specific activity: 3.8 MBq/mg; Anisole, specific activity: 30.27 MBq/mg; Benzoic acid, specific activity: 17.01 MBq/mg (~ 3.3 - 75 µg/mL); All purchased from American Radiolabeled Chemicals (St. Louis, USA).
Determination of the radioactivity: Radioactivity of liquid samples was measured by liquid scintillation counting with automatic quench correction by the external standard channel ratio method at 13 °C using Ultima Gold® high flashpoint scintillation cocktail (Packard Instrument BV Chemical Operations, Groningen, The Netherlands).
HPLC-^{14}C-detection: The employed columns had the following dimensions: 125 x 4.6 mm, 125 x 2 mm, 125 x 1 mm with quick connector and 125 x 0.8 mm, 125 x 0.5 mm, 125 x 0.3 mm capillary columns. All columns were filled with GROM-Saphir 110, C18, 5 µm material. The ^{14}C-detection cells had volumes of 30 µl, 8 µl, 3 µl and 1.5 µl. The injection volume was always kept proportional to the cross section of the employed HPLC column and therefore also proportional to the employed flow.
Two HPLC systems were employed. The first one consisted out of a Micro Pro® HPLC pump (Eldex, Napa, USA), an Endurance® Auto sampler (Spark, Emmen, Netherlands), and a Mistral Slave® column oven (Spark, Emmen, Netherlands).
The second one was a capillary LC-System 1100 (Agilent, Waldbronn, FRG) consisting of Degasser, CapPump, Micro ALS, ALS Therm, and a Mistral Slave® column oven (Spark, Emmen, Netherlands).
HPLC-^{14}C-MS/MS: The chromatographic equipment was analogous to first system described above. This system was connected to an API 3000 triple stage mass spectrometer with an electrospray source (Sciex, Toronto, Canada). The flow was split past the column at different ratios, leaving a flow of 5 µl/min for the mass spectrometer while the remaining flow was led into the radioactivity monitor.
Microsomal incubations: The final substrate concentration was 20 µM, while the protein concentration was 1 mg/ml. The substrate was incubated for 3 hours at 37 °C and the reaction was terminated by addition of acetonitrile.
Radioactivity monitor: Ramona 2000 connected to HP-DOS workstation (raytest GmbH, Straubenhardt, FRG) and HP 35900 A/D-converter C (Hewlett-Packard, Waldbronn, FRG).

Results

The scintillation material particle size did not play an important role for the signal intensity (peak height as well as peak areas) using glass scintillator. Four different particle sizes were investigated in an identical cell volume (Figure 1) and did not show a difference being greater than the standard deviation. To our impression, minor differences in the cell assembly like dead volumes from connectors and capillaries have a far greater influence than the particle size of the scintillation material. These findings are of practical use since greater particles produce not only lower back pressures which is important for the combination with mass spectrometry where a split is needed, but do also lessen the risk of detector contamination due to their smaller surface.

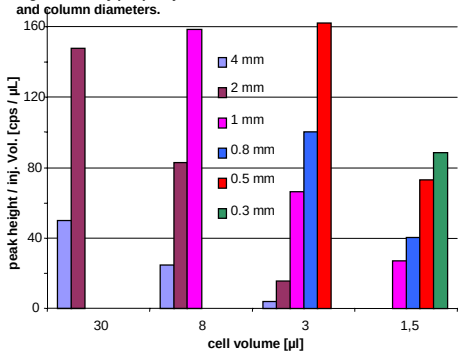
A comparison of the signal intensities per sample volume with different column diameters and flow cell volumes is given in Figure 2. While the S/N ratio in UV-detection should theoretically increase by the factor of 60 on changing from 4.0 mm to 0.5 mm columns (using the same detection cell), an increase by a of factor 40 was observed in ^{14}C-detection (using a 3 µl cell). In UV-detection as well as in ^{14}C-detection, the detection cell volume should be adjusted adequately to the column cross section , for keeping good resolution [1]. Nevertheless, downscaling of UV-detection is expected to give by the following reasons better results than downscaling of ^{14}C-detection: ^{14}C-detection is time-dependent, while the UV signal depends - following Lambert-Beer's law- on the path lengths, which allows to minimize the cell volume on keeping the path length almost constant. A reduction of the ^{14}C-cell volume reduces the analyte's residence time in the cell,

Figure 1:
Signal intensity (peak height) with identical detection cells having different scintillation material particle sizes.



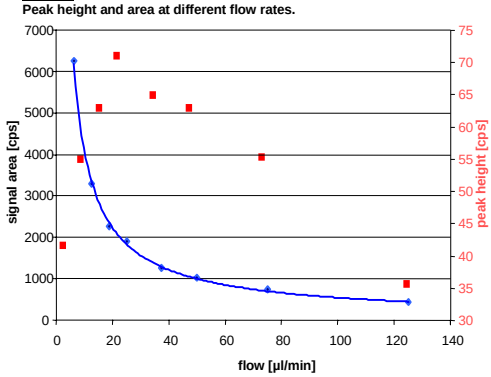
therefore the counting time and so the signal intensity. The influence of the residence time is also shown in Fig. 3, wherein the residence time is determined by the flow. A severe gain in signal intensity is also reported from Capillary LC-MS/MS-detection, from which an increase of factor 30 was shown for a change of 2 mm against 180 µm columns and a factor of even 700 for a change of 4.6 mm against 75 µm columns [2, 3].
The best signal intensities per sample volume were achieved with a 0.5 mm column and a 3 µl flow cell. A gain of factor 3 in sensitivity per sample volume compared to the 4 mm column with a 30 µl detection cell was observed. These data implicate that Capillary LC-^{14}C-detection provides an ideal solution for small sample volumes containing high specific activities, while for higher diluted radioactive samples greater column diameters are suggested due to their higher loading capacity.

Figure 2:
Signal intensity per µL injection volume at different flow cell volumes and column diameters.



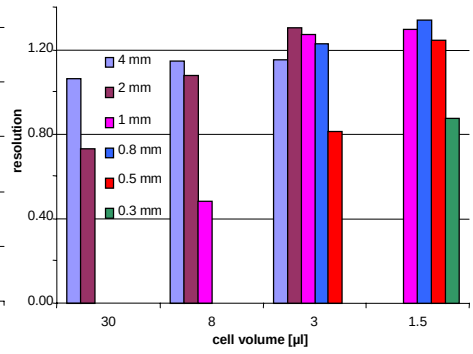
To investigate the time dependence of the ^{14}C-signal intensity, a volume of 2.5 µl was injected onto a 1 mm column with a 3 µl flow cell at increasing flow rates. The peak areas decrease exponentially with a rising flow rate (Figure 3, blue line), indicating that the analyte's residence time in the detection cell is responsible for the peak area.
The maximum peak height, however, is reached close to the optimal flow rates, due to the minimization of the longitudinal diffusion. At flow rates higher than 30 µl (corresponding to 1 mm/s), however, the peak height decreases as well, but less dramatically than the peak areas, due to shorter residence time in the detection cell (Figure 3, red squares).

Figure 3:
Peak height and area at different flow rates.



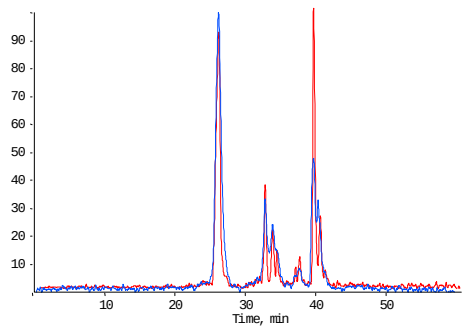
Capillary HPLC columns provide higher chromatographic resolution, provided that the employed detection cells have a sufficiently small volume. Radio detection however, requires cell volumes which seem rather large compared to those employed in UV or fluorescence. In Figure 4 changes can be seen at the 2 mm and the 0.5 mm columns, while a significant improvement - by factor 3 - is observed at a 1 mm column, on reducing the cell volume from 8 to 3 µl. As a general tendency, Figure 4 shows, that a further reduction of the detection cell volume does not necessarily result in a better resolution is observed in the case of the 4 mm, the 0.8 mm and the 1 mm column for cell volumes below 3 µl.

Figure 4:
Resolution at different cell volumes and column diameters.



The practical effect of the decreased cell volume on the chromatographic resolution is shown Figure 5. The use of HPLC columns with inner diameters of 300µm -or even smaller- resulted in too low signals in the mass flow-dependent ^{14}C-detector.

Figure 5:
Metabolite profiles in incubations of a drug candidate with liver microsomes on a 1mm column with a 8 µl detection cell (blue line) and a 3 µl detection cell (red line).



Conclusions

Capillary columns were found to be very suitable, provided that the radioactivity is not too diluted in the sample.

- No influence of the scintillation material particle size on the signal intensity was found. However, small particles tend to produce a higher back pressure or even blockage of the detection cell. Further on, they increase the risk of cell contamination due to their greater surface. Cell contamination ends easily up in an increase of the background, worsening the signal to noise ratio.
- The best signal intensities per sample volume were achieved with a 0.5 mm column and a 3 µl flow cell. A gain of factor 3 in sensitivity per sample volume compared to the 4 mm column with a 30 µl detection cell was observed.
- Flow velocity has a great influence on the peak area as well as on the peak height. While lowest flow rates result in large peak areas, higher flow rates are required for better peak shape (S/N ratio) and acceptable analysis times.
- Chromatographic resolution improves by decreasing the detection cell volume, but is paid by lower signal intensities. Optimal combinations were found to be: 2 mm column/ 30µl cell, 1 and 0.8 mm columns/3 or 8µl cell; 0.5 mm columns/3 µl cell.
- Chromatography with 0.8 mm i.d. columns proved to be suitable in regard of robustness, reliability and reproducibility of the analysis. The low sample consumption per chromatographic run and the simultaneous combination of MS and MS/MS scanning allowed an almost automated screening of small sample amounts.

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References

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