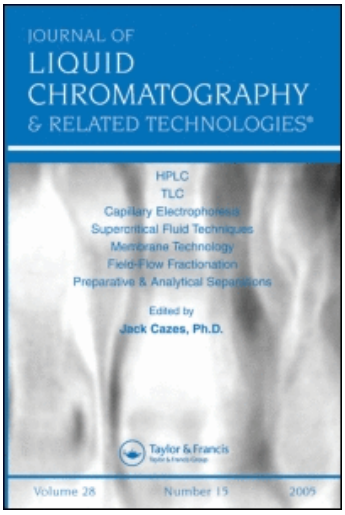

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Radioactivity Detectors for High-Performance Liquid Chromatography in Drug Metabolism Studies

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Abstract: Sensitive radioactivity detection following high performance liquid chromatography (HPLC) remains a challenge in drug metabolism studies with radiolabeled compounds. The aim of this review article is to give a general overview of scintillation counting techniques and to summarize recent advances in radioactivity detection for HPLC, with a strong emphasis on the application in drug metabolism studies. State of the art and new developments with homogeneous and heterogeneous on-line radioactivity detectors, stop-flow technology, off-line microplate solid scintillation counters (SSC), and accelerator mass spectrometry (AMS) will be discussed.

Keywords: Accelerator mass spectrometry, Heterogeneous counting, Homogeneous counting, HPLC, Microplate solid scintillation counting, Radioactivity detection

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INTRODUCTION

Biotransformation or metabolism is a biochemical process by which undesirable compounds (xenobiotics) such as pesticides, industrial chemicals, pollutants, secondary metabolites from plants or moulds are structurally converted by enzymes in order to be quickly eliminated from the body. Biotransformation also controls the fate of desirable compounds like drugs, endogenous compounds, or vitamins in the body. The beginnings of xenobiotic metabolism are closely connected to the work of Alexander Ure in the UK and Wilhelm Keller in Germany. They did the first human metabolism studies in 1841–1842 observing the conversion of benzoic acid to hippuric acid, a conjugate with glycine (a phase II metabolite), by analyzing urine.[1] At that time, characterization of metabolites was done by isolating the compounds from body fluids with subsequent structure elucidation by elemental analysis, derivatization procedures and synthesis of metabolite references.

Approximately one century later, the basic principles of metabolism and the classification into phase I or phase II metabolites were introduced.[2]

With the increased interest in the behavior of compounds, which were applied to the body of animals or humans, the technologies to analyze these compounds and their degradation products in biological materials evolved. Brodie et al. developed methodologies for quantitation of basic organic compounds in biological material using newly developed detection technologies such as ultraviolet spectroscopy.[3,4] Soon after the discovery of the radioactive isotope carbon ^{14}C, experiments to study biochemical pathways were initiated.[5] It became evident that the administration of radiolabeled xenobiotics offered the possibility to follow the fate of xenobiotics quantitatively as well as qualitatively. The first studies dealt with the distribution of 3,4-benzpyrene-5-^{14}C and 1,2,5,6-dibenzantracene-9,10-^{14}C in mice, including the characterization of the metabolic degradation products,[6] the distribution of radioactivity in rats after administration of radiolabeled 2-acetylaminoflourene[7] and the distribution and excretion of radioactivity in rats receiving *N*-methyl-^{14}C-erythromycin.[8] At that time, determination of radioactivity was performed with so-called internal flow counters or nucleometers after combustion of the respective samples (off-line liquid scintillation counting).[9,10] Metabolite patterns were generated by using paper chromatography or thin layer chromatography (TLC) and visualized by applying radioautography,[11,12] gas radioanalysis (anthracene scintillation),[13] and, later, gas–liquid chromatography.[14]

Due to the continuous improvements in newly developed forms of chromatography, and the combination with new methods of detection,

chromatography in general and more specifically high-performance liquid chromatography (HPLC) became the most important tool for the generation of metabolite patterns and quantitation of metabolites. The general name for all forms of chromatography in combination with radioactivity detection is simply radiochromatography.[15]

Commercially flow-through radioactivity detectors became available from the second half of the last century onwards.[16] Flow-through detectors can be used either in the liquid scintillation counting (homogeneous) or in the solid scintillation counting (heterogeneous) mode. As described in this review below, flow-through detectors have some advantages in comparison to the off-line counting techniques with regard to handling and technical complexity.

Drug metabolism and pharmacokinetic (DMPK) studies are essential requirements for the registration of new chemical entities. Since carbon and hydrogen are typically part of organic compounds, the method of choice to follow the fate and quantity of a drug and its metabolites *in vitro* or *in vivo* is still radiolabeling, either by ^{3}H or ^{14}C.[17] Both ^{3}H and ^{14}C are relatively weak β^{-}-emitters and therefore relatively easy to handle with regard to radiation safety. Other β^{-}-emitters such as the radioisotopes ^{33}P, ^{32}P or ^{35}S and in some cases the γ-emitter ^{125}I can also be used. Even positron emitters with very short half lives, such as ^{13}N, were used to investigate the metabolism of xenobiotics.[18] The radiolabel can be introduced at either one position or at several different positions in the molecule. If two different radioisotopes are introduced into the molecule it is called dual-labeling. Radiolabels, which ideally do not affect the physicochemical properties of the compound and which should be metabolically stable, allow both the qualitative and quantitative determination of the parent compound and its metabolites. With the radiolabel as a marker it is much easier to distinguish the precursor compound and its labeled metabolites from endogenous matrix compounds to finally create metabolic profiles. The use of radiolabeled drug candidates is therefore highly accepted and implemented in FDA, EPA and ICH guidelines.[19] The interest in metabolism of new drugs and the role of metabolites as potential toxic agents is still increasing. Preclinical and clinical animal and human metabolism studies using labeled material become more and more important for the investigation of human-specific metabolites and the quantitation of metabolite levels for the safety evaluation process.[20,21]

The aim of this review article is to give a general overview of scintillation counting techniques and to summarize recent advances in radioactivity detection for HPLC, with a strong emphasis on the application in drug metabolism studies including metabolite profiling and identification. The following types of radioactivity detectors will be described in detail: (a) homogeneous and heterogeneous flow-through monitor

analyzer systems, (b) stop-flow technology, (c) microplate solid scintillation counters (SSC), and (d) accelerator mass spectrometry (AMS).

PRINCIPLE OF SCINTILLATION COUNTING

Independent of the nature of radioactive decay (β^-, β^+, γ or α irradiation) radioactivity can be measured and quantified by scintillation counting. In this process radioactive irradiation is measured indirectly by transforming its energy into a directly proportional amount of visible energy, i.e., photons. This emission of photons by the scintillation material is known as fluorescence and can be monitored by a photomultiplier (PMT). Scintillation counting can be done by using either a solid[22,23] or a liquid[24,25] scintillation material. In liquid scintillation counting (LSC) the radioactive sample is mixed with a cocktail containing a solvent and an organic scintillator. The solvent molecules transfer the energy of the irradiation to the fluorescent molecules which produce light with a particular wavelength ($\lambda = 325$–450 nm). Solvents used, such as for instance diisopropylnaphthalin or phenylorthoethane are optically transparent to avoid absorption of the emitted light and, for safety reasons, should neither be toxic nor show a high flash point. As organic scintillators, 2,5-diphenyloxazole can be used and, if necessary, the wavelength of the emitted photons can be extended by so-called secondary scintillation molecules such as 1,4-di-(2-5-phenyloxazolyl)-benzene. Nowadays scintillators such as 1-phenyl-3-mesityl-2-pyrazoline are used which emit light at $\lambda = 430$ nm without the need for secondary scintillators. Scintillators used in LSC have to meet the following requirements: (a) the yield of photon production has to be very high at a short fluorescence period, (b) the range of emission should fit to the spectral sensitivity of the PMT, (c) the scintillator has to be well soluble in the organic solvent, (d) it should be compatible with the sample, aqueous solutions and additives such as solubilizers, (e) it should have a very low self-absorption, (f) and it should be relatively insensitive to quenching compounds. Since the radioactive sample is mixed homogeneously with the scintillator molecules, LSC has been preferably used to measure low energy β^--emitters such as ^{3}H which do not have a high penetration range in liquids or solid matter. For β^--emitters such as ^{14}C or ^{32}P or γ-emitters, solid scintillation counting can be used. Although in this case the radioactive sample is more distant from the scintillation material than with LSC, the energy of the β^--irradiation is high enough and the interaction of γ-irradiation is low enough to reach and penetrate the solid scintillation material for photon generation. Typical solid scintillators are NaI and lithium glass.

The light emitted from scintillators is detected by a PMT which consists of a photocathode to produce ideally one electron from a single photon impact, a series of dynodes to produce an electron cascade (secondary emission) for signal amplification and an anode to create the signal output. Since PMTs are sensitive to light the scintillation material has to be shielded from other sources of light or radioactivity to reduce the background. The electronic and thermal noise from the whole instrument also have an impact on the background. Background which is produced by incidental light registration can be reduced by using two PMTs in coincidence. With this arrangement only photons arriving in both PMTs simultaneously within a defined time frame in the range of 200 ns are measured, thereby monitoring only real scintillation events. A multichannel analyzer allows to determine the number of photons and the energy of these photons. The result is a typical energy spectrum for the measured radioisotope.

An alternative counting principle, as applied in microplate SSC (see below), is based on single PMT, time-resolved pulse discrimination.[25] This is a counting technique that provides low background levels and high sensitivity. It uses one PMT to count radioactivity in a sample, which facilitates close physical alignment of multiple PMTs for simultaneous counting of several samples as in microplates. Scintillators with long decay periods are used. Photons will be emitted over a long time period, until all of the absorbed energy has been released. This means that each decay event produces a packet of photons followed by a series of pulses, whereas PMT noise consists of single pulses. If the initial packet is followed by one or more additional pulses in a certain time window, the pulse probably belongs to a true decay event as depicted in Figure 1a. Otherwise, the initial pulse is apparently background and is rejected, as shown in Figure 1b. If multiple pulses that exceed the threshold are counted – two or three pulses are sufficient to distinguish them from background noise – the decay event is regarded valid and further analyzed in the pulse height analyzer as in conventional LSC.

For both scintillation counting approaches – either with a two PMT setup or with one PMT, time-resolved pulse discrimination – energy spectra and efficiency might be compromized by an effect called quenching. Various reasons for quenching exist: (a) physical quenching due to the reduction or elimination of the contact between the radioactive compound and the scintillation cocktail, for instance due to tissues or phase separation between liquids, (b) chemical quenching due to the addition of other chemicals to the cocktail which are not similar to the solvent in the cocktail, and (c) color quenching due to the absorption of the emitted light by dyes.

Due to the possible existence of quenching, the real number of disintegrations per minute (dpm) might be higher than the measured number

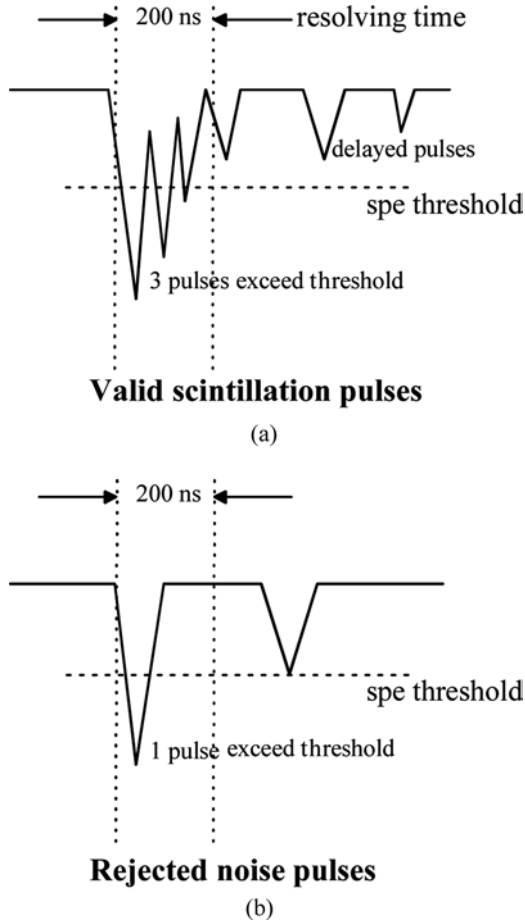

Figure 1. Principle of time-resolved pulse discrimination. (a) True decay event, (b) rejected decay event.

of counts per minute (cpm) in the scintillation counter. The cpm-to-dpm-ratio is called counting efficiency. To obtain dpm from cpm, a quench correction can be performed. Different methods for quench correction can be applied to compensate this adverse effect.[25] A summary of quench correction methods is outside the scope of this review.

Radioactivity detection depends basically on the following characteristics: the counting time, the counting efficiency, and the background. A general equation for the limit of detection (*LOD*) is:

$$LOD = \frac{271}{T \times E} + \frac{46.5\sqrt{B}}{\sqrt{T \times E}} \quad (1)$$

where T is the counting time in minutes, E is the counting efficiency as a percentage and B is the background radioactivity in dpm.[26] Obviously, changes in one of the three parameters B, E, or T effect the LOD. In most drug metabolism studies, radioactivity concentrations may be low, and with a low number of disintegrations N, the relative uncertainty becomes large. A logical way to improve the relative uncertainty is to increase the counting time T such that a sufficiently large N is obtained. With low counts near background, an additional source of error is the statistical uncertainty σ_{BG} of the background counts N_{BG}. From the total measured counts N_{total} (with a statistical uncertainty σ_{total}), the background count N_{BG} is subtracted, to give the net sample counts N_{sample}. Due to the propagation of errors, the uncertainty σ_{sample} is then given by Equation (2):[27]

$$\sigma_{sample} = \sqrt{\sigma_{total}^2 + \sigma_{BG}^2} = \sqrt{N_{total} + N_{BG}} \tag{2}$$

Starting with Equation (2), an alternative equation for LOD can be derived as done by Plesch:[28]

$$LOD = 3\sqrt{\left(R_{BG}\left(\frac{1}{T_{sample}} + \frac{1}{T_{BG}}\right)\right)} \tag{3}$$

where R_{BG}, T_{sample}, and T_{BG} are the background counting rate, sample counting time and background counting time, respectively.

As with Equation (1), Equation (3) is also a pragmatic approximation for practical use.[29]

The other option to lower the LOD is to either increase counting efficiency E or to decrease background radiation B. In general, E depends on the isotope used, the sample composition as well as on the characteristics of the type and method of the scintillation counter. B can be minimized by the use of scintillation cocktails with preferred properties resulting in less quenching and by affecting physical parameters (i.e. pre-cooling of samples, avoiding scattered light).

Basically, all examples of the recent developments in radioactivity detection as described in this review deal with improvements of one of the parameters in Equation 1 or combinations thereof.

TYPES OF HPLC RADIOACTIVITY DETECTORS

On-Line Radioactivity Detection

In the last century, radioactivity detection was used in conjunction with paper chromatography,[30] TLC,[31] gas chromatography,[32] and supercritical fluid chromatography.[33] This section will focus on on-line

radioactivity detection coupled to HPLC, because it is the most widely applied chromatographic method in drug metabolism studies.

In 1955, Bangham investigated the metabolism and excretion of ^{14}C-labeled diethylcarbamazine primarily by using paper chromatography and off-line counting of LC fractions and a homemade on-line radioactivity detection cell.[34] He outlined that although the off-line approach is sensitive and quantitatively relatively accurate the on-line approach would be less laborious and faster. In the following twenty years peak resolution and counting efficiency improved due to optimized cell designs, new scintillation materials, homogeneous and heterogeneous counting, lower background and better solvent compatibility.[35–43]

The first coupling of a radioactivity detector to HPLC was performed by Sieswerda and Polak.[44,45] Using different solid scintillation materials incorporated into a U-shaped on-line detection cell, they compared off-line and on-line radioactivity detection. Theoretical considerations revealed how the precision in on-line counting depends on the sample count rate, background count rate and the residence time in the cell. In 1977, Hodenberg investigated metabolic profiles in biological matrices by direct injection of urine and bile on a reversed-phase C_{18} HPLC column which was connected to an on-line radioactivity detector.[46] In the following years further comparison of both off-line and on-line counting were performed and further optimizations in on-line detection were introduced to increase counting efficiency and peak resolution.[47–52] Due to the favorable properties of HPLC and on-line radioactivity detection, this combination developed into a routinely applied method for the investigation of metabolic profiles.[5,53–60] The configuration of an on-line radioactivity detector in HPLC is similar to that of standard scintillation counters with the exception that the vial is replaced by a coiled tube positioned between two PMTs. The general setup of an HPLC system coupled to an on-line radioactivity detector and mass spectrometer is shown in Figure 2. The simplest setup in case of heterogeneous counting only consists of an HPLC (1), a UV/VIS detector (2) and a radioactivity detector (3) (Figure 2a). For on-line homogeneous counting, a scintillation cocktail (8) has to be added to the HPLC eluent by means of a pump (7) and a T-piece (6) (Figure 2b). For both counting modes, on-line mass spectrometric detection for structure elucidation can be applied simultaneously by adding a further splitter (4).

As already mentioned, the *LOD* can be improved by increasing the counting time T which can be done by prolonging the residence time in the detection cell. Several attempts were made to increase the residence time in on-line radioactivity detection. Veltkamp et al. obtained sensitivities comparable to off-line counting by using post-column solvent segmentation.[57] Hexane was added to the aqueous effluent to create

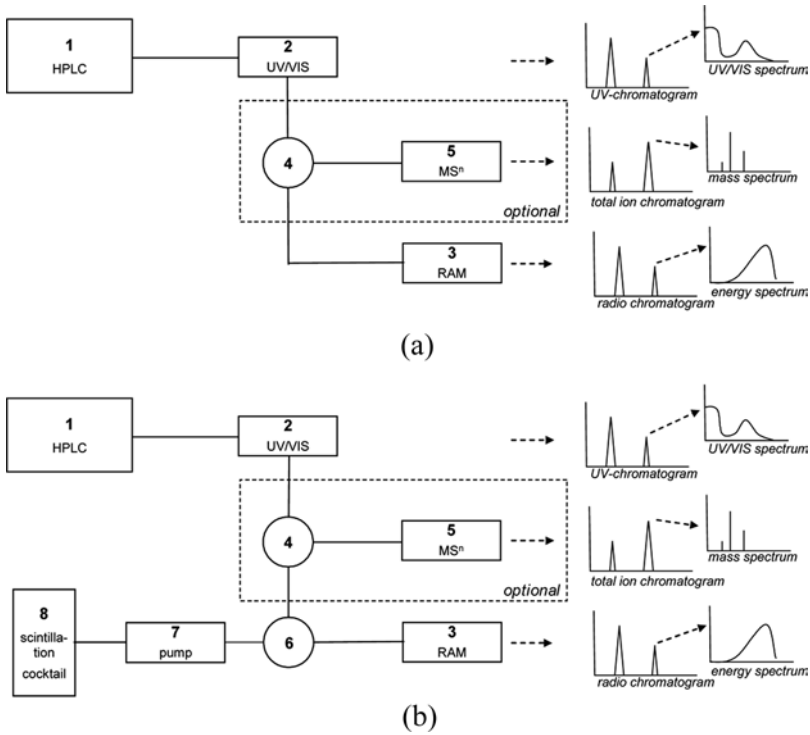

Figure 2. Schematic set up for on-line HPLC-radioactivity detection with optional on-line mass spectrometric detection for structure elucidation. (a) Heterogeneous counting, (b) homogeneous counting.

segments which were stored in a loop. After separation and standard on-line counting the segments were re-introduced through the radioactivity detector by using a slower flow rate. 17-fold higher sensitivities could be obtained compared to the standard on-line approach. However, this method is limited to eluents containing solvents which are not miscible with hexane. Furthermore the heterogeneous scintillation cell which was used showed low counting efficiencies. The same group investigated the metabolites of urapidil with increased sensitivity by extracting the compounds into a water-immiscible liquid scintillator using ion-pair extraction.[58,59] The extracted and segmented compounds were stored in a loop and "regions of interest" could be measured again for a longer period to enhance the sensitivity by re-injection into the detection cell.

Another approach to increase sensitivity was reported by Baba et al.[61] A so-called synchronized accumulating radioisotope detector (SARD), in which five detector cells filled with solid scintillation material

were connected in series for signal accumulation, was tested. This system was superior to a single counting cell and could achieve an *LOD* of 10 Bq. A similar on-line radioactivity detection cell with four cells connected in series is available from the company Raytest and was specifically designed for micro-HPLC.[62]

By downscaling to nano- and micro-HPLC injected sample amounts can be reduced and sensitivity increased. However, with regard to peak resolution and counting efficiency the standard on-line radioactivity detection cells are normally not compatible to micro- or even nano-flow rates. Schultz and Alexander incorporated a solid scintillation radioactivity detection cell into a micro column to perform on-column radioactivity detection.[63] By using silanized lithium glass as scintillation material they investigated the influence of the scintillator particle size and of the cell volume on peak broadening. They found that the particles of scintillator should be similar in size and shape as the particles of the stationary phase in HPLC and that always a trade-off between sensitivity and peak resolution needs to be made. Whole-column radioactivity detection where all of the stationary phase is located in the detector volume was described by Link and Synovec.[64] A much simpler approach is to split the solvent stream coming from the miniaturized HPLC column and to add a make-up flow which is compatible with standard on-line radioactivity detectors.[65,66]

Several publications dealt with a direct comparison of on-line heterogeneous and homogeneous scintillation counting. These comparisons showed that each mode has its own advantages and disadvantages,[49,50,52,60,67] as summarized in Table 1. Heterogeneous counting has favorable aspects with regard to consumable costs, sample recovery and safety whereas homogeneous counting still seems to be superior with regard to sensitivity and counting efficiency. The latter are key in metabolism studies, in particular if ^{3}H-labeling is involved.

Another approach to increase counting time is a stop-flow system as implemented in the so-called LC-ARC (liquid chromatography–accurate radioisotope counting) system. It was originally invented by Lee et al. and has been commercialized by the Aim Research Company in 1998.[68] Basically the LC-ARC system consists of a liquid chromatograph, a radioactivity detector, a stop-flow controller and an ARC™ data system. The core of the system is the stop-flow controller, which is able to control HPLC systems and radioactivity detectors from different suppliers (i.e., Agilent, Waters, PerkinElmer, Raytest, Berthold). All components can be operated by the LC-ARC software. The LC-ARC system is able to work (a) by stopping the chromatographic flow at predefined time intervals, (b) by stopping the flow triggered through levels of radioactivity and (c) by continuously monitoring radioactivity as in traditional on-line flow-through monitors. A schematic sophisticated setup

Table 1. Comparison between homogeneous and heterogeneous on-line scintillation counting. The plus sign indicates a relative advantage compared to the other mode based on literature data

	On-line scintillation counting	
Requirements	*homogeneous*	*heterogeneous*
Sensitivity	+	
Counting efficiency (e.g. ^{3}H)	+	
Background	+	
Decontamination	+	
Sample recovery		+
Chemical quench		+
Re-use of scintillation material		+
Impact of solvent composition on efficiency		+
Impact on peak resolution		+
Applicability to biomolecules	+	
Waste management		+
Consumable costs		+
Use of hazardous liquids		+

with inclusion of mass spectrometric detection and fraction collection can be found in Figure 3. The LC-ARC system can be easily coupled to mass spectrometers for on-line structure elucidation of metabolites and direct

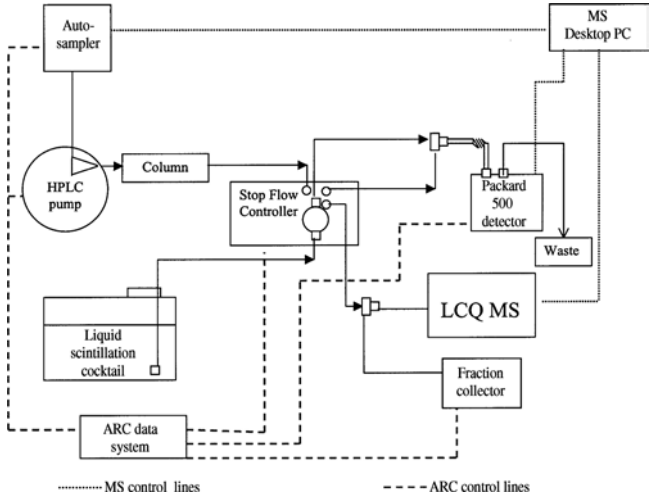

Figure 3. Experimental setup with stop-flow controller, radioactivity detector, mass spectrometer and fraction collector.[26]

assignment of radioactivity to the respective metabolites.[69] By splitting the LC-flow only a small portion of the HPLC eluent will be directed into the mass spectrometer whereas the remainder will reach the radioactivity detector operated by the LC-ARC system. The LC-ARC can work in the stop-flow technique to trap the radioactive peaks in the radioactivity detector in order to apply a longer counting time and, thus, to enhance sensitivity and accuracy. In the peak-parking technology the flow rate is slowed down if radioactivity has reached the detector which allows either more preferential radioactivity counting statistic but also longer acquisition times for full scan mass spectrometry as well as special experiments such as parent ion scan or MS^n. Not only sensitivity and peak resolution are enhanced but also volatile metabolites can be investigated with full recovery.

LC-ARC systems typically reach an *LOD* in the range of 5–20 dpm for ^{14}C and 10–40 dpm for ^{3}H and is therefore approximately 10–20-fold more sensitive than flow-through detection systems either in the homogeneous or in the heterogeneous scintillation mode. The *LOD* can be easily decreased in the LC-ARC system by using optimized stop-flow modes and counting times, although the counting time seems to reach plateaus after five to ten minutes of counting time.[56]

Applications of the LC-ARC system are described for biological samples from *in vivo* animal drug metabolism studies and for *in vitro* metabolism studies. Further application areas are within the field of environmental fate studies or for quality control after synthesis of radiolabeled compounds. Performance, precision and accuracy of the system were tested for all available modes.[56]

Figure 4 shows an HPLC-ARC chromatogram of an unknown labeled drug substance from human urine. Conditions applied are shown in the legend. The *LOD* was 12 cpm under optimized conditions and is therefore about 4-fold less sensitive compared to off-line SSC, which will be described below.

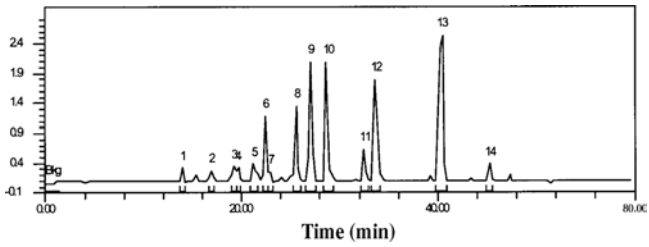

Figure 4. Radiochromatogram showing the ^{14}C-labeled compound A and metabolites in human urine resulting from optimized stop-flow techniques using the LC-ARC system. *LOD*: 12 cpm; fraction interval: 15 s; counting time: 2 min.[26]

Off-Line Microplate Solid Scintillation Counting

The first report on the use of off-line microplate solid scintillation counting (SSC) as a new mode of off-line radioactivity detection for HPLC appeared in 2000.[70] Nowadays, this off-line detection method has been embraced as a convenient way for high sensitivity analysis of HPLC fractions in drug metabolism studies with radioactively labeled compounds in several pharmaceutical companies.

As already described in the previous sections, the use of conventional on-line radioactivity detectors is limited by their relatively low sensitivity, although this can be enhanced by working in the stop-flow mode. Off-line solid scintillation counting of HPLC fractions collected in solid scintillator-coated 96-well microplates using a microplate scintillation counter is very well suited as a sensitive radioactivity detection method for various (miniaturized) separation techniques like HPLC and capillary electrophoresis (CE).[70] Compared with classical off-line LSC as a high-sensitivity off-line HPLC radioactivity detection principle, SSC in the microplate format enables a significant reduction in laboratory work and increases sample throughput. Besides that, it is more sensitive than LSC, which allows to use smaller sample amounts. The general setup for the fractionation of small volumes eluting from an HPLC column or CE capillary is simple. The eluent fractions are automatically dispensed into opaque microplates using a fraction collector. In case of microbore HPLC, with flow rates from approximately 5 to 50 μL/min and column inner diameters between 0.3 and 1 mm, a special microfraction collector has to be used or a make-up flow must be added to obtain a sufficient droplet formation rate. After collection of the fractions into the microplates, the plates are dried in a Speedvac evaporator, whereafter the individual wells can be counted quickly. By plotting the number of counts for each well (i.e. fraction) versus the retention time (i.e., total run time divided by total number of wells times the well number) a graph can be created which represents the off-line radiochromatogram.

So far, two papers have been published that present a thorough validation with respect to sensitivity, accuracy, precision and radioactivity recovery of microplate SSC using Lumaplates in combination with the TopCount instrument as an off-line HPLC radioactivity detector in drug metabolism. Microplate SSC was compared with the conventional off-line LSC.[29,71] In both publications, it was concluded that SSC detection was 2- to 3-fold more sensitive than LSC and that the relative abundance of metabolites in biological matrices can be determined with comparable accuracy and precision as with LSC. Besides that, biological matrices like plasma, blood, urine, bile and feces had no or only small effects on the quantitative analysis of ^{14}C-labeled compounds. Practical examples in

several ADME studies showed very similar radiochromatograms monitored by off-line SSC compared with off-line LSC. Figure 5 nicely illustrates the influence of counting time and the counting method, SSC or LSC, on the signal-to-noise-ratio. In this particular example, the amount of radioactivity injected was only about 600 dpm. Significant reduction of the baseline noise, both with SSC and LSC, was observed. The background levels and background fluctuations are considerably lower with SSC than with LSC. The mean of the background is about 1.8 cpm with SSC and about 12 dpm with LSC. The improved

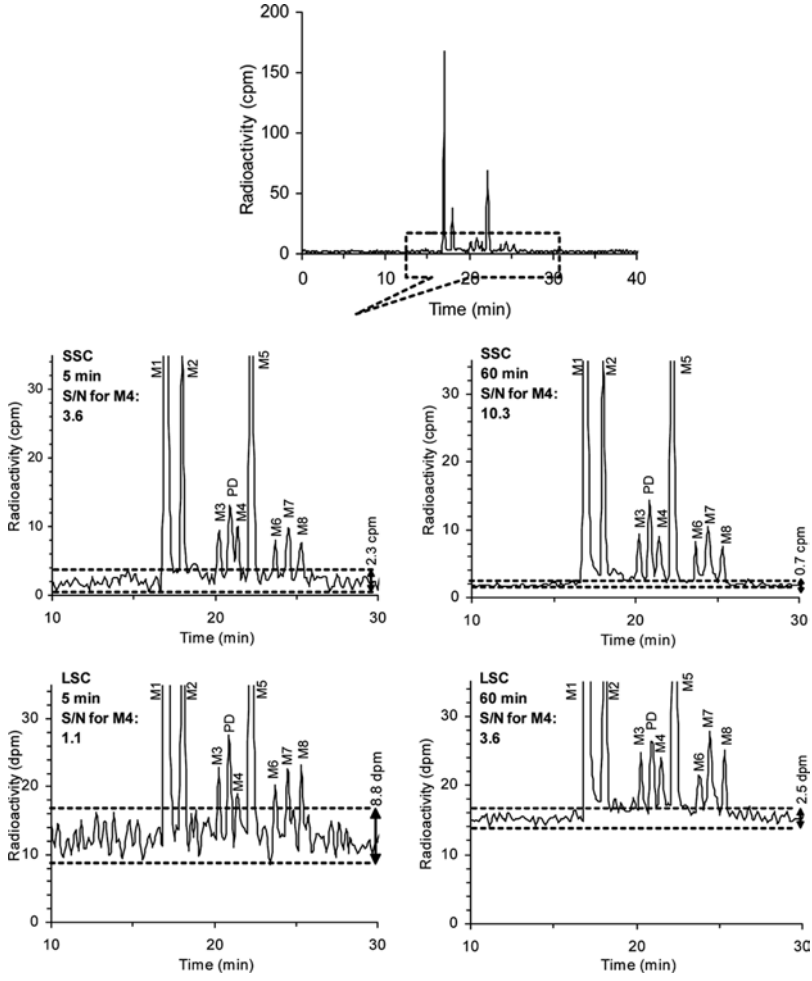

Figure 5. Influence of counting time on signal-to-noise ratio with off-line LSC and SSC detection.[29]

background characteristics of microplate SSC detection lead to approximately 3.5 times better signal-to-noise-ratios, compared with off-line LSC. A comparable signal-to-noise-ratio of 3.6 for the minor metabolite M4 was obtained for SSC at five minutes counting time per well and LSC at 60 minutes counting time per vial. With the latter counting method a total counting time of more than eleven days was necessary whereas the same sensitivity was achieved with SSC within a few hours total counting time. Thus, counting in several wells simultaneously results in a substantial increase in throughput, compared with classical sequential LSC. Both papers conclude that SSC is very well suited as an alternative tool for HPLC profiling of radioactive metabolites.

A paper published by Nassar et al. described a direct comparison of metabolite patterns in biological matrices, which were obtained by detection with LC-ARC and off-line SSC under similar HPLC conditions.[69] The results showed a higher sensitivity and lower detection limit with SSC detection whereas the total run time of LC-ARC was approximately half that of off-line SSC detection. It was regarded as an advantage that LC-ARC detection does not need manual operations such as sample transfer and data import. Also the risk of loosing volatile metabolites does not exist with LC-ARC detection.

The ability of TopCount SSC to act as a suitable radioactivity detection system for ultra-performance liquid chromatography (UPLC) was evaluated recently.[72] The introduction of robust small particle packed columns and the availability of commercial instrumentation for UPLC is of special interest for application in drug metabolism studies. A smaller particle size increases the separation efficiency and sensitivity which enhances the peak capacity per unit time. Therefore, the use of on-line radioactivity detectors is very limited in combination with UPLC due to the large volumes of the homogenous counting cells employed. Since very small peak widths of about three seconds are obtained by UPLC, in a 2.1 I.D. $\times$ 100 mm column filled with 1.7 μm particles, the total dwell time per UPLC fraction was reduced to about 2.3 seconds, which is considerably shorter than the normally used fraction collection times of 7–15 seconds. With a flow rate of 300 μL/min, this corresponded to peak volumes of approximately 10 μL per fraction. Under these conditions, the radioactivity for each analyte was contained within 2–3 wells. It was found that the robustness and reproducibility of the UPLC-TopCount SSC detection was not compromised by repeated injection of crude biological matrices such as extracts of feces, plasma and bile.

Another example of the combination of a miniaturized separation technique and SSC is the combination of CE and TopCount detection. Figure 6 shows electropherograms of a ^{14}C-labeled drug in water, used as an application solution in an animal study (unpublished results). UV-detection, TopCount detection and MS detection were applied.

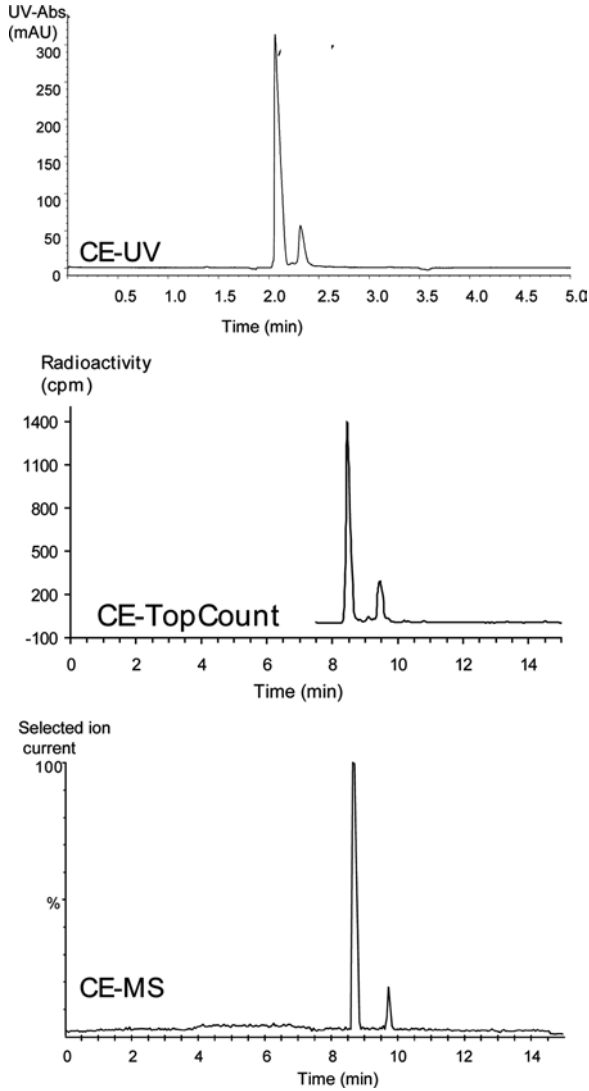

Figure 6. Capillary electrophoresis of a ^{14}C-labeled drug with UV-detection, off-line SSC radioactivity detection and MS detection. Capillary:75 µm I.D.; 90.0 mm total length; Background electrolyte: 40 mM ammonium acetate, 0.1 mM EDTA, pH 7.6. Dynamic coating: flush with BGE plus 0.01% (w/v) HDB (polybrene). Make-up liquid in case of SSC detection: BGE/methanol (90/10, v/v), 320 µL/min→one droplet/well.

In case of SSC TopCount detection, the fraction collection time was 3 s/well to reduce the loss of separation power. The radiolabeled impurity, migrating behind the main peak, was nicely separated and visualized with all three modes of detection.

The application of narrow-bore HPLC and TopCount SSC for samples from crop metabolism studies, such as tomato extracts or wheat grain, or samples from farm animal metabolism studies, such as goat urine samples or extracts of hen excreta was described by Kiffe et al.[73] These samples could be injected, analyzed and directly quantified without any further sample pre-treatment. It was clearly demonstrated that the combination of HPLC with TopCount SSC was superior to on-line radioactivity detection and at least equivalent to classical TLC regarding sensitivity.

The work described above in this section was all conducted using the TopCount technology with LumaPlates for the collection of the column effluent after HPLC separation. It is well established now that the collection of HPLC fractions into LumaPlates is the most sensitive radioactivity detection method nowadays for routine metabolism studies with radiolabeled compounds. With off-line SSC, it would be of interest to recover the radioactivity from the LumaPlates after counting for subsequent structure elucidation by spectrometric methods. However, this is hard to achieve due to limited solubilization of the radioactivity from the solid yttrium silicate scintillator at the bottom of the wells, whereas the extracts are also highly contaminated with scintillator fixing compounds.

Alternative 96-well plates/counting systems have also been tested during the last years. For instance, metabolite profiling and identification by fractionation into 96-well Scintiplates followed by MicroBeta radioactivity counting was described.[74] It was demonstrated that isolated metabolites could be further analyzed by mass spectrometry which therefore allowed metabolite quantitation and structure elucidation from a single analysis. For excreta samples, quenching of the radiochemical signal by endogenous matrix components made the Scintiplates approach inappropriate for metabolite quantitation of ^{14}C-labeled material for all species investigated. However, plasma samples could be quantified in a reliable and robust fashion.

A second alternative is the use of Cytostar-T Plates. These microplates are standard 96-well plates, in which the transparent bottom of each well is a proprietary homogenous mixture of polystyrene and scintillants. They can be counted in the TopCount microplate counter. Although background levels were higher and the sensitivity about 3-fold lower than with LumaPlates, similar signal-to-noise ratios as with LSC can be achieved. MS analysis of isolated metabolites extracted from the Cytostar-T plate was possible with high sensitivity, whereas it was

simultaneously demonstrated that MS analysis of metabolites extracted from LumaPlates was basically impossible.[75]

A very recent paper addressed the development of optimal strategies for the combination of radioactivity detection and MS detection for metabolite quantitation and structure elucidation, in *in vivo* ADME studies.[76] In the so-called one-dimensional approach, biological samples are analyzed by HPLC/MS using exactly the same HPLC conditions as for the generation of metabolite patterns with a radioactivity detector for quantitation of metabolites, either by on-line counting or by off-line microplate SSC. Increased sensitivity for MS is obtained by down-scaling the analytical conditions to micro- or nano-flow rates. As stated in this paper, this approach only works for major metabolites, whereas concentrations can be too low in case of minor and trace metabolites. The two-dimensional approach, which consisted of (a) separation of metabolites in a normal-bore HPLC column, (b) subsequent fractionation and identification of radioactivity by microplate SSC in wells of 96-well plates, (c) re-injection of separated metabolites on microbore HPLC columns combined with simultaneous off-line SSC and MS/MS, allowed not only to quantify metabolites, but also to characterize a wide range of trace metabolites at very low concentrations. Due to the reduced amount of co-eluting matrix components, the signal-to noise-ratio could be increased significantly with the two-dimensional approach. The still available fractions from the first dimension enable the re-analysis of specific metabolites in additional experiments like hydrogen/deuterium exchange experiments or product ion scanning experiments.

Accelerator Mass Spectrometry (AMS)

AMS is a technology used for the measurement of rare isotopes and originates from the 1970's. Actually this technology has been developed for carbon dating. Its application to radiochemical radiotracer studies started in 1990.[5]

AMS differs significantly from the other methods described in this review. AMS is able to detect very low levels of enriched ^{14}C with high precision; therefore, very small sample quantities are needed.

The following sample preparation steps are necessary for AMS measurements: (a) conversion of the biological sample to graphite, (b) transfer and pressing the graphite into a cathode and (c) placing the cathode into the AMS ion source. From the AMS ion source, negatively charged ions are generated to be transferred after pre-selection into a so called tandem electrostatic Van der Graaff particle accelerator. In the tandem accelerator the ions finally loose the outer valency electrons, and with the loss of the electrons, the charge of the ions changes from negative

to positive charge state (from 1^+ to 4^+). The positive ion beam exits the tandem accelerator and before reaching the detector, stable isotopes and competing isotopes need to be removed ($^{12}C/^{13}C$). Due to the low abundance further focusing of the ^{14}C ion beam is needed for final counting in a gas ionization detector. A detailed and more comprehensive description of the AMS method is subject of several publications.[5,77–81]

The *LOD* of AMS in biological samples depends on the carbon content and therefore on the type of sample investigated. The *LOD* in AMS for plasma is approximately 0.6 dpm/g. A full metabolic profile can be generated by injection of less than 0.1 dpm onto the HPLC column.[5]

AMS technology can be applied to all studies where radiolabels are currently used including pharmaceutical research and development, cancer research, endocrinology and pharmacology.[78] Consequently, applications of AMS to the full pre-clinical and clinical ADME package, including mass balance, pharmacokinetics and metabolism studies is an interesting option. AMS can be effectively used to obtain early ADME/PK data in microdosing studies, which can be regarded as pre-regulatory human phase 0 studies.[79] Lappin and Garner presented data from a typical absolute bioavailability study where an extravascular, therapeutic nonlabeled dose is administered simultaneously with an intravenous [^{14}C]-microdose (3.7–7.4 kBq). Plasma concentrations of the orally administered drug were determined by LC-MS, whereas the plasma concentrations of the intravenously administered drug resulted from HPLC followed by AMS. The metabolite profile was generated by injecting a plasma extract onto the HPLC column (in total 0.07 dpm). The column effluent were collected into fractions and afterwards analyzed individually by applying the AMS technology to reconstruct the entire chromatogram. The *LOD* can be roughly estimated to be less than 0.001 dpm.[79]

HPLC-AMS profiles from radiolabeled drug and metabolites in urine as well as extracts of feces and plasma from the farnesyl transferase inhibitor [^{14}C]R115777 resulting from a human mass balance and metabolism study showed the parent drug and five metabolites (Figure 7). All HPLC separations were obtained after injection of less than 5 dpm. The HPLC-AMS chromatograms were qualitatively and quantitatively very similar to those obtained after LC-MS/MS. In this human phase I study the administered radioactive dose was at least 1,000-fold lower than that needed for conventional radioactive studies.[80]

With respect to radiation safety and environmental protection the AMS technology offers clear advantages compared to standard studies using radiolabeled drug compounds. Moreover the drug development process can be significantly accelerated in case the microdosing approach or a cassette dosing approach in human are taken into consideration.

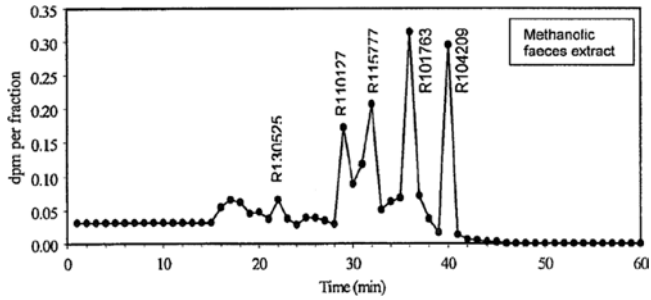

Figure 7. Example of metabolite profile of a combined pool of methanolic extracts of feces samples generated from injecting less than 5 dpm on the HPLC column. Fractions were collected every minute to be analyzed by AMS.[80]

Clear limitations of AMS for a widespread use are the intensive and time consuming sample preparation procedure for the graphitization process prior to the analysis, special laboratory requirements to avoid radioactive contamination and the high costs. Further improvements to facilitate these preparatory steps are already under consideration.[77]

CONCLUSIONS

Drug metabolism studies are essential requirements for the registration of new drugs. The quality of drug metabolism studies using radiolabeled compounds requires highly sensitive and selective instruments for the generation of metabolite patterns and the detection of single metabolites even at trace levels. Several new techniques have been developed in radioactivity detection during the mid of the last century. Despite of some disadvantages, such as additional efforts to reduce radiation safety issues and radioactive waste, lower stability due to radiolysis, and additional costs for radiosynthesis, it can be expected that due to the high sensitivity and selectivity of radioactivity detection, highly sophisticated techniques in LC such as HPLC or UPLC combined with radioactivity detection will continue to play a major role for identification and quantitation of metabolites in animal and human ADME studies.

Depending on the radioisotope and the concentration of radioactivity in the respective sample matrix, the metabolism chemist has to choose the most suitable mode of radioactivity detection. Samples with high concentration of radioactivity and a low matrix contamination should preferably be analyzed with an on-line flow-through radioactivity detector. In case of sufficiently high concentrations, a flow cell with a solid scintillator and heterogeneous counting can be applied instead of homogeneous counting in order to reduce waste and consumable costs.

The heterogeneous counting mode is approximately 10 times less sensitive compared to the homogeneous counting approach (*LOD* approximately 60 dpm versus 600 dpm for heterogeneous counting, assuming a typical HPLC flow rate of 1 mL/min). A further gain in sensitivity can be reached by using the LC-ARC system. In the stop-flow mode this system is able to detect approximately 12 dpm in 1 min fractions of an analytical HPLC run. All on-line methods described here are fully automated and do not require any sample manipulation for the counting process itself. However, high amounts of radioactive waste are produced and the analytical systems need to be decontaminated from time to time.

Off-line microplate SSC and AMS are the most recently introduced technologies. Microplate SSC is especially suited for low concentrations of radioactivity as in human ADME studies or in case a sample is highly loaded with endogenous matrix compounds preventing the injection of sufficient amounts of radioactivity into the HPLC system. Depending on the counting time, the plate type used and the instrument applied an *LOD* of 1 dpm can be easily reached. However, it should not be forgotten that the total counting time for a single HPLC run might take more than 24 hours.

The AMS detection principle differs significantly from the other methods described here. It is an off-line radioactivity detection method and can be regarded as the most labor and cost intensive technology. It has its advantages in case of extremely low concentrations of radioactivity and for special ADME/PK studies (i.e., micro dosing).

A trend towards a further miniaturization in analytical separation methods has been observed during the last fifteen years which ultimately lead to the development of the so-called lab-on-the-chip systems. Radioactivity detection in such extremely miniaturized systems, which would find an interesting field of application in drug metabolism studies, will require completely new miniaturized and automated detection cell designs. This is probably valid for both off-line and on-line counting methods.

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