Journal of Chromatography B, 868 (2008) 49–57

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Utilizing a −100 °C microplate CCD Imager, yttrium silicate coated 384-microplates and ultra-performance liquid chromatography for improved profiling of radiolabeled drug metabolites in complex biological samples

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ARTICLE INFO

Article history:
Received 8 February 2008
Accepted 13 April 2008
Available online 13 May 2008

Keywords:
UPLC
Drug metabolism
ViewLux
LumaPlate-384
Metabolite profiling

ABSTRACT

The recent commercial availability of small particle packed columns (<2 μm) and associated instrumentation capable of withstanding the high pressures of such columns, has lead to an increase in the application of so called ultra-performance liquid chromatography. The improved efficiency, resolution and peak capacity of these columns, when coupled to mass spectrometry, provides particular benefit for the identification of drug metabolites in complex biological samples. In this work, the ability of ViewLux™, a microplate imager, to act as a suitable radiodetection system for ultra-performance liquid chromatography methods is assessed. The system demonstrates robustness and sensitivity comparable to a microplate scintillation counter (TopCount®) more typically used for off-line metabolite radiodetection. The ViewLux is also used here to undertake successful metabolite profiling of actual samples, for two investigational drug candidates, using both 96- and 384-well yttrium silicate microplates.

1. Introduction

As part of the development of novel drug candidates, it is important to gain an understanding of the disposition of the drug molecule in humans and animals used in toxicological safety testing. Traditionally, this is accomplished with a range of *in vitro* and *in vivo* studies, which are broadly described as ADME studies (*A*bsorption, *D*istribution, *M*etabolism, *E*xcretion). This work is typically performed with radiolabeled material, to act as a tracer to help identify and isolate (if necessary) drug metabolites, to demonstrate absorption and distribution, and to determine the routes of excretion. Importantly, the use of radiolabel also facilitates the quantification of drug and metabolites in complex samples such as excreta and tissues, necessary to help define any species differences. Although high-performance liquid chromatography (HPLC) coupled to on-line radioactivity flow detectors (RFD) has been successfully used to separate and detect radiolabeled drug metabolites, the recent trend to smaller particle chromatography (<2 μm) has lead to an increase in ultra-performance liquid chromatography (UPLC) methods in these applications [1–3]. It has been demonstrated in recent articles that UPLC methods, with inherent robustness, efficiency and resolution can be used for drug metabolite separation and identification, when coupled with fast scanning mass spectrometers [1–3]. The further coupling of UPLC to microplate scintillation counters (MSC), such as TopCount, has also been reported, thereby providing methods for both identification and quantification using off-line scintillation counting [4].

The coupling of MSCs, such as TopCount to UPLC offers high sensitivity and high resolution, compared to the more traditional approach using HPLC with RFDs. The narrow peak widths and longer counting times available from UPLC combined with TopCount provide a more sensitive method of profiling drug metabolites in complex biological samples, particularly when samples contain low concentrations of drug-related material [4]. MSC systems rely on increased counting times for sensitivity (typical count times for MSC are 2–5 min per well, compared to RFD cell residency times of 7–10 s) and are currently the only means of reliable radiodetection for high efficiency UPLC separations. However, this results in an inherent delay between injecting the sample and being able to view and process the data, which can especially hinder method development.

In this work, a microplate imager (ViewLux) is evaluated as an alternative to TopCount and similar MSC systems, for off-line radiodetection of UPLC separations. Unlike TopCount which uses 12 internal photomultipliers (PM-tubes), ViewLux microplate imaging relies on a back illuminated CCD (charged-coupled device) camera operating at −100 °C coupled to an optimised telecentric lens, which enables whole microplates to be imaged in one. This

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doi:10.1016/j.jchromb.2008.04.013

is in stark contrast to PM-tube based detection which can only measure one well per PMT at a time. The camera and lens systems operating in the luminescence detection mode are suitable for radiometric assays. The ViewLux therefore should possess all the advantages of traditional MSCs, such as enhanced sensitivity and lack of hazardous liquid scintillant (*cf.* RFDs), but also provide quasi-instantaneous data, which is independent of plate well density. Recent applications have compared the ViewLux to PM-tube based MicroBeta® and VICTOR^{2TM} detectors for luminescence, fluorescence, photometry and time-resolved fluorescence analyses [5,6]. Although these PM-tube based instruments are still regarded as high throughput (HT), the ViewLux due to the ability to image entire plates is best described as ultra-HT. The ViewLux is already an established assay platform for scintillation proximity assays (SPA) in biology screens and other similar HT applications [7–9]. In this work, the ViewLux has been evaluated as an off-line counting device, for detecting and quantifying drug metabolites separated by UPLC, using microplates with both 96- and 384-well formats. The results obtained by UPLC with ViewLux are compared here to an established MSC platform (TopCount), in terms of peak detection, sensitivity and precision.

2. Experimental

2.1. Chemicals and materials

HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Loughborough, UK). Analytical ammonium acetate was purchased from BDH (Poole, UK). De-ionised water was generated in the laboratory using a Millipore Mill-Q water filter unit (Molsheim, France). [^{14}C] and non-radiolabeled drug-1 and [^{14}C] and non-radiolabeled drug-2 were synthesised by GlaxoSmithKline Research and Development, Stevenage, UK. [ring-U-^{14}C] tolbutamide (1.85 MBq/mL in ethanol), [2-^{14}C] chlorzoxazone (1.85 MBq/mL in ethanol) and S-[4-^{14}C] mephenytoin (1.85 MBq/mL in ethanol) were supplied by Amersham Biosciences (Bucks., UK). [8-^{14}C] caffeine (3.7 MBq/mL in ethanol), bovine serum albumin, glucose and Kreb's Ringers bicarbonate buffer were supplied by Sigma–Aldrich Co. (Dorset, UK). [^{14}C] Sucrose (NEC-100X, 3,7 MBq/mL) in ethanol/water was supplied by PerkinElmer. Control human plasma and bovine red blood cells were obtained from GlaxoSmithKline Research and Development, Ware, UK. Sprague-Dawley CD rats were supplied by CharlesRiver, U.K. Ltd. (Inveresk, UK).

2.2. Preparation of standard solutions

Approximately 20 μL aliquots of [^{14}C] tolbutamide, [^{14}C] chlorzoxazone and [^{14}C] S-mephenytoin, together with a 10 μL aliquot of [^{14}C] caffeine, were dispensed into a scintillation vial and diluted to 5 mL with 50 mM ammonium acetate (pH 5). To confirm the level of radioactivity in the standard mixture, 20 μL aliquots were counted in triplicate by liquid scintillation counting (Beckman model LS6000IC, Beckman Coulter UK Limited, High Wycombe, Bucks., UK), following the addition of 10 mL of Starscint scintillation fluid (PerkinElmer, Pangbourne, Berks, UK). This confirmed that the radioactive concentration was approximately 8000 DPM per analyte. This stock solution was used for spiking all samples as detailed below. Additional dilutions were made in buffer, where appropriate, to provide suitable low-level DPM solutions for analysis.

For preparing the dilution series of [^{14}C]-sucrose, 3 μL from the [^{14}C]-sucrose stock solution was first added to 6.66 mL of Milli-Q water (∼100 DPM/μL). This was then used for preparing the dilutions of 76.8; 38.4; 19.2; 9.6; 4; 2.4; 1.2; 1; 0.8; 0.6; 0.4 and 0.2 DPM/μL.

2.3. Preparation of spiked samples

Samples were generated by spiking known amounts of the standard solutions into control human plasma extract to produce final concentrations of ∼10 DPM/μL for each analyte. Plasma extractions were conducted as described below.

2.4. Sample extraction

Control human plasma was extracted by vortex-mixing aliquots (2 mL) for approximately 1 min with two volumes (4 mL) of acetonitrile. The samples were sonicated for 1 min in an ultrasonic bath and rotary-mixed for approximately 30 min. The extracts were then centrifuged at approximately 1500 × *g* at 4 °C for 10 min. The supernatant was removed, the pellets were re-suspended in acetonitrile (4 mL) and the process repeated. The supernatants were combined and evaporated under nitrogen to dryness and reconstituted in 1 mL water/methanol (v/v, 50/50).

2.5. Samples from an in situ liver perfusion experiment

A rat liver was isolated and perfused using a standard perfusion protocol [internal reference]. The perfusate was prepared using washed bovine red blood cells (haematocrit approximately 30%), bovine serum albumin (3%, w/v), glucose (0.1%, w/v) and made up to a total volume of 150 mL with Kreb's Ringers bicarbonate physiological buffer. Under isoflurane anaesthesia, the hepatic portal vein, bile duct and superior vena cava were cannulated, and major blood vessels ligated to isolate the liver from the periphery. The liver remained *in situ*, and was connected to the perfusion apparatus, such that the perfusate entered through the hepatic portal vein and exited through the superior vena cava. As the perfusate was circulated, it was continuously warmed to 37 °C, filtered and oxygenated using humidified 95% oxygen and 5% carbon dioxide.

Radiolabeled (1.97 mg) and non-radiolabeled drug-1 (13.71 mg) were dissolved in a total volume of 1552 μL of DMSO (dimethyl sulfoxide) and 250 μL was then added to the circulating perfusate. Bile was collected pre-dose and at 0–4 h after dosing, into pre-weighed containers and stored at approximately −20 °C when not in use.

A second rat liver was isolated and perfused using the procedure outlined above. Radiolabeled (4.9 mg) and non-radiolabeled drug-2 (17.9 mg) were dissolved in a total volume of 2000 μL of DMSO (dimethyl sulfoxide) and 250 μL was then added to the circulating perfusate. Bile was collected pre-dose and at 0–4 h after dosing, into pre-weighed containers and stored at approximately −20 °C when not in use.

2.6. UPLC

All UPLC separations were performed on a Waters Acquity UPLC^{TM} system (Waters, Manchester, UK), with an upper pressure limit of *ca.* 1000 bar [10], and equipped with a binary pump, an autosampler and a tunable UV detector. Injections of standards and spiked human plasma extracts were made onto a 100 mm × 2.1 mm i.d., 1.7 μm Waters Acquity BEH C_{18} column (Waters, Manchester, UK) operated at 40 °C, using a mobile phase of 50 mM ammonium acetate, pH5 (A) and methanol (B). The proportion of B was programmed to linearly increase from 20% to 47% over 1.58 min, with a further increase to 50% at 3.87 min. At 5 min, B was ramped up to 90% and then returned to 20% at 5.5 min. The column was then allowed to re-equilibrate for approximately 2 min prior to the next injection. All analyses were performed at a flow rate of 0.3 mL/min. UPLC fractions were collected into 96-well microplates (Shallow-well LumaPlate®-96, PerkinElmer LAS (UK) Limited, Beaconsfield, Bucks., UK) or 384-well microplates (deepwell LumaPlate®-384,

PerkinElmer LAS (UK) Limited) at a rate of ~2 s/well using a modified CTC HTX Pal fraction collector (CTC Analytics, Zwingen, Switzerland). Control of the Acquity UPLC™ was through Masslynx (v4.1) software (Waters, Manchester, UK).

For the UPLC analysis of rat bile (5 μL), from an isolated perfused rat liver, following administration of [^{14}C] drug-1, separations were performed with a mobile phase of 20 mM ammonium acetate, pH 5 (A) and acetonitrile:methanol (7:3) (B). The proportion of B was programmed to linearly increase from 25% to 50% in 8.0 min, with a further increase to 100% at 9.5 min. The proportion of B was then held at 100% until 11.0 min and then returned to 25%. The column was then allowed to re-equilibrate for approximately 1.0 min prior to the next injection. All other conditions are as described above.

For the UPLC analysis of rat bile (5 μL), from an isolated perfused rat liver, following administration of [^{14}C] drug-2, separations were performed on a 100 mm × 2.1 mm i.d., 1.7 μm Waters Acquity BEH Phenyl column (Waters, Manchester, UK) operated at 50 °C with a mobile phase of 20 mM ammonium acetate, pH 7 (A) and acetonitrile:methanol (85:15) (B). The proportion of B was programmed to linearly increase from 20% to 75% in 10 min, with a further increase to 90% at 12 min. The proportion of B was then returned to 20%. The column was then allowed to re-equilibrate for approximately 1.5 min prior to the next injection.

2.7. TopCount

After collection, all LumaPlate-96 microplates were dried in an oven at 60 °C or a vacuum hub and then either sealed using microplate heat sealing film (TopSeal-S, PerkinElmer LAS (UK) Limited) or left unsealed. Radioactivity (CPM value) of the dried residues in the 96-well plates was determined using a TopCount NXT (PerkinElmer LAS (UK) Limited), typically with a counting time of 5 min. Prior to counting, the TopCount was calibrated, the 12 detectors were normalised, and the backgrounds of the photomultipliers were determined. The counting results were stored as ASCII files. The ASCII files were converted into reconstructed radiochromatograms for integration using the import function of Laura software, version 3.3 (Lablogic, UK) and were also reconstructed into radiochromatograms for visual inspection using XP Excel (Microsoft Corporation, USA).

2.8. ViewLux

The ViewLux (PerkinElmer, Waltham, MA) was used for whole-plate light imaging, using the CCD Imager in luminescence detection mode. The ViewLux was generally set to measure luminescence for either 300 or 600 s using 12 × 12 binning (96-well microplates) or 8 × 8 binning (384-well microplate) and a dark adaptation time of minimally 30 min. All ViewLux data is presented as ADUs (analogue to digital units), a number relative to the number of incident photo-electrons. An important difference between PMT counters and non-PMT instruments is that PMT counters like TopCount use photon counting which is expressed as CPM, while non-PMT instruments use relative light units (RLU). ADU is also a relative unit depending on the setting of the gain. Furthermore, since this is an imager, ADU values are calculated as the average value of all pixels in the well.

The counting results were stored as ASCII files. The ASCII files were converted into reconstructed radiochromatograms for visual inspection using XP Excel (Microsoft Corporation, USA).

3. Results and discussion

In previous work, the authors illustrated the advantages of using UPLC coupled with MSC systems (such as TopCount) for metabolite separation and quantification [4]. This relied on optimisation of a fast post column fraction collector, to preserve the high chromatographic performance, and at the same time adequately deliver collected fractions into 96-well microplates (Shallow-well LumaPlate-96), for radiometric analysis on TopCount. This approach generated typical chromatograms using 3 × Shallow-well LumaPlate-96 plates. The high resolution chromatography was maintained providing an inherently sensitive method of profiling drug metabolites in complex biological samples [4]. However, the disadvantage of this off-line approach for radiometric analysis is the significant time between injecting the sample and processing the data, compared to on-line RFDs which offer instantaneous data readout. With only 12 PM-tubes (TopCount NXT), counting one microplate row at a time, this culminates in 2 h total counting time, using a 5 min count per row (×3 microplates). If this is multiplied over the course of an entire metabolism study, which may include the analysis of 30–50 samples from different matrices (e.g. plasma, bile, urine, etc.), it becomes evident that the time it takes to inject and chromatograph metabolites using UPLC is disproportionate with the time it takes to detect these metabolites. This disparity is also true of HPLC based methods, coupled to MSC systems, but is accentuated when using UPLC, where analytical run times are typically ≤10 min, compared to ≤60 min for HPLC.

3.1. Initial evaluation

To evaluate the plate imager approach for off-line radiometric analysis of isolated drug metabolite fractions, the linearity, precision and sensitivity of the ViewLux was assessed using standard Shallow-well LumaPlate-96 microplates, spiked with increasing levels of [^{14}C]-sucrose: 3, 6, 9, 12, 15, 18, 36, 59, 144, 288, 576 and 1151 DPM (n = 5). A microplate was spiked (15 μL per well) and dried in a vacuum hub prior to measurement. A plot of the mean ViewLux ADU *vs.* the DPM spiked per well indicates that ViewLux provides a linear response across the radioactivity range tested, R^2 = 0.999 (see Fig. 1). These data are tabulated in Table 1.

The limit of detection for the ViewLux, using a 5 min counting time and [^{14}C]-sucrose, was determined by the standard approach, defined as the mean of the background corrected three times by the blank standard deviation ($y = y_B + 3s_B$, where y is the signal with minimum detectable analyte concentration, y_B is the signal of the blank, and s_B is the standard deviation of the blank), using the least squares method to transform the data from ADU to DPM. Blank wells (H1–H12) in the [^{14}C]-sucrose spiking microplate were used to determine the background measurement (n = 12). This provides a limit of detection for ViewLux of approximately 3 DPM, which

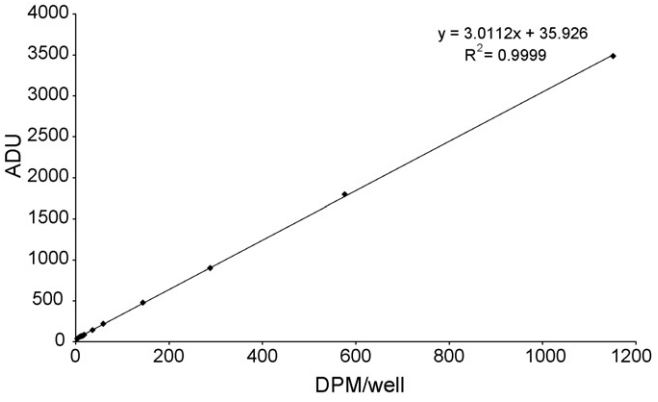

Fig. 1. A plot of ViewLux™ ADU response *vs.* DPM: LumaPlate-96 microplate [^{14}C] sucrose serial spiking (n = 5).

Table 1
ViewLux ADU *vs.* DPM (spiked Shallow-well LumaPlate-96)

DPM	Background subtracted ADU[a]	STDEV	%CV
1151	3452	73	2.1
576	1763	31	1.7
288	867	13	1.4
144	444	12	2.5
59	185	8	3.4
36	109	8	5.6
18	50	3	3.7
15	39	3	4.4
12	34	3	4.1
9	26	2	2.6
6	14	3	5.8
3	5	2	3.8

[a] Mean of $n = 5$ (background = 35 ± 2 ADU).

is comparable to previously published values for TopCount and MicroBeta [11–14]. However, since radiodetection, as detailed in this application, is fundamentally used to quantify metabolites in animal and human samples, a more appropriate measure of detection is the limit of quantification. Calculated to be approximately 7 DPM using this system, based on accepted rational, $y = y_B + 10s_B$, again using least squares method to transform the data from ADU to DPM. Since the ViewLux is an imager, ADU values are calculated as the average values of all pixels in the well. Therefore, even if low values are observed, detection is based on the total counts in a well divided by the number of pixels in a well. Hence, low values can have extremely good statistics, and hence low limits of detection, because measurement is determined on many total counts.

3.2. ViewLux vs. TopCount

Standard solutions of [^{14}C]-caffeine, tolbutamide, chlorzoxazone and *S*-mephenytoin were used to compare ViewLux and TopCount response, within a chromatographic context. Injection of standard mixtures (100 and 500 DPM per analyte), were made onto the UPLC system and fractions collected into 3 × Shallow-well LumaPlate-96 microplates as outlined in Section 2. Dried and sealed plates were counted on the TopCount and ViewLux, using standard protocols. The exposure time for ViewLux plate imaging was 10 min per microplate and the count time for TopCount was 5 min per microplate row ($n = 12$ per row). This difference equates to a total count/image time of 30 min using ViewLux and 120 min using TopCount (3 × plates), and serves to demonstrate the superior throughput of the CCD image-based system *vs.* the PM-tube based system. The fact that the ViewLux can image entire plates at once means the exposure time can be significantly reduced to increase the congruity between off-line detection and UPLC separation. A comparison of reconstructed radiochromatograms for TopCount and ViewLux are shown in Fig. 2 for 500 DPM per analyte on-column. The data are almost comparable, with all components clearly discernible, although using this plate format and standard measurement protocols the signal-to-noise is superior on the TopCount chromatograms. The analysis was repeated with spiked human plasma extract (Fig. 3) to check for matrix effects from protein, salt, or other endogenous material, often associated with biological samples, as well as chemical quenching. Similar data was obtained for 100 DPM per analyte standards and spiked plasma (data not shown). Examination of the raw ASCII file from each UPLC ViewLux chromatogram demonstrated that the majority of the radioactivity for each analyte resided in 2–3 wells, thereby maximising the DPM collected per fraction, while maintaining the chromatographic resolution. This combination of efficient separation, fast fraction collection and static counting time, provides the

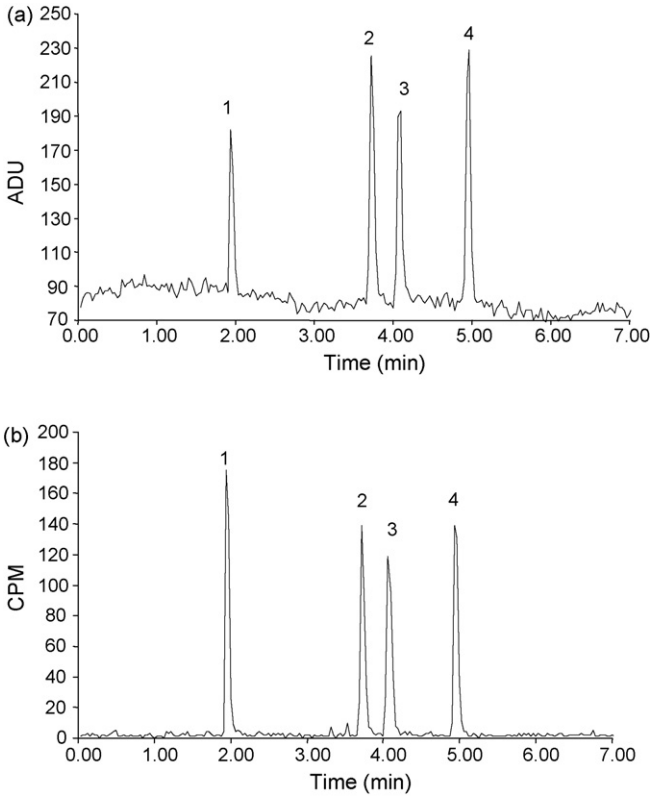

Fig. 2. Reconstructed radiochromatograms following UPLC of standard mix (LumaPlate-96 microplates): (a) ViewLux and (b) TopCount (1 = caffeine, 2 = *S*-mephenytoin, 3 = chlorzoxazone, 4 = tolbutamide, *ca.* 500DPM per analyte on-column, 50 μL injection). Fraction collection speed ~2 s/well.

UPLC/TopCount or ViewLux system with superior sensitivity (*cf.* HPLC with RFDs) [4].

As TopCount consists of 12 independent PM-tubes in the same instrument, this can represent a possible source of inaccuracy, due to variation in response between different photomultipliers, resulting in variable data being observed in wells emitting the same amount of light. This is usually negated by normalisation against a reference plate. Despite this, statistical errors between individual PM-tube readings can still occur, contributing to instrument noise. Imager systems like the ViewLux do not suffer these problems, as light from all wells in the plate is received and focussed simultaneously by the telecentric lens, which removes any parallax error (i.e. no shading). In addition, noise is further reduced by minimising thermal noise in the instrument, by operating the CCD chip at −100 °C. Thereby dark noise is significantly reduced. The low variation of ViewLux measurements was assessed by spiking five separate Shallow-well LumaPlate-96 microplates with [^{14}C]-sucrose as outline above, which were then imaged (10-repeats per microplate). At all DPM per well values examined (see Table 2), the inter-plate precision values (CV%) were always less than 3.5%, and are therefore acceptable, indicating extremely low variation in discrete repeated measurements.

The intra- and inter-plate (five plates, 10 plate repeats) variation of background was also very low with negligible difference between background readings across five plates (mean ADU range 34–37). To achieve low background and high precision results, plates are optimally kept in the dark some time before measurements (dark adaptation). The LumaPlates reached background after 30 min, indicating the required dark adaptation time to achieve the best sensitivity (data not shown).

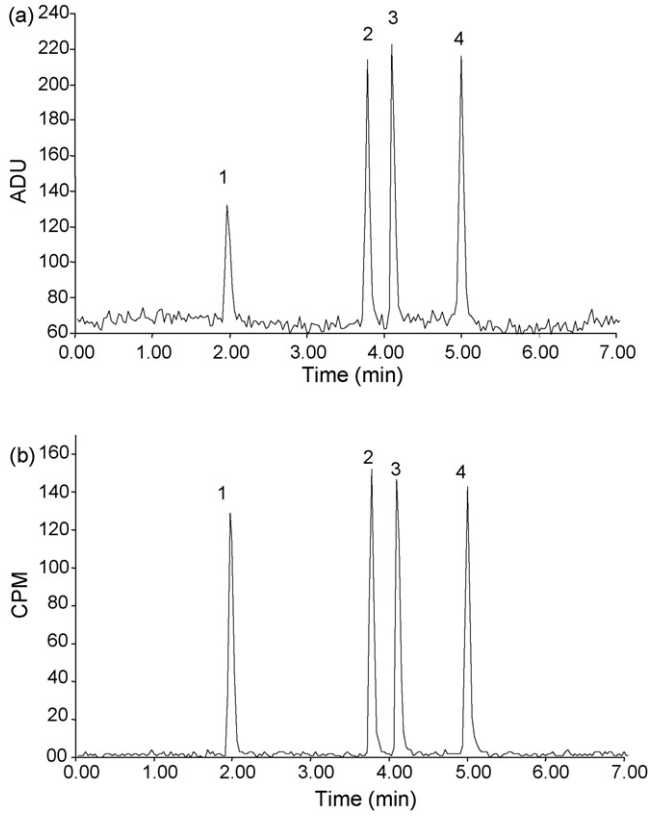

Fig. 3. Reconstructed radiochromatograms following UPLC of spiked human plasma extract (LumaPlate-96 microplates): (a) ViewLux and (b) TopCount (1 = caffeine, 2 = *S*-mephenytoin, 3 = chlorzoxazone, 4 = tolbutamide, *ca.* 500 DPM per analyte on-column, 50 μL injection). Fraction collection speed ~2 s/well.

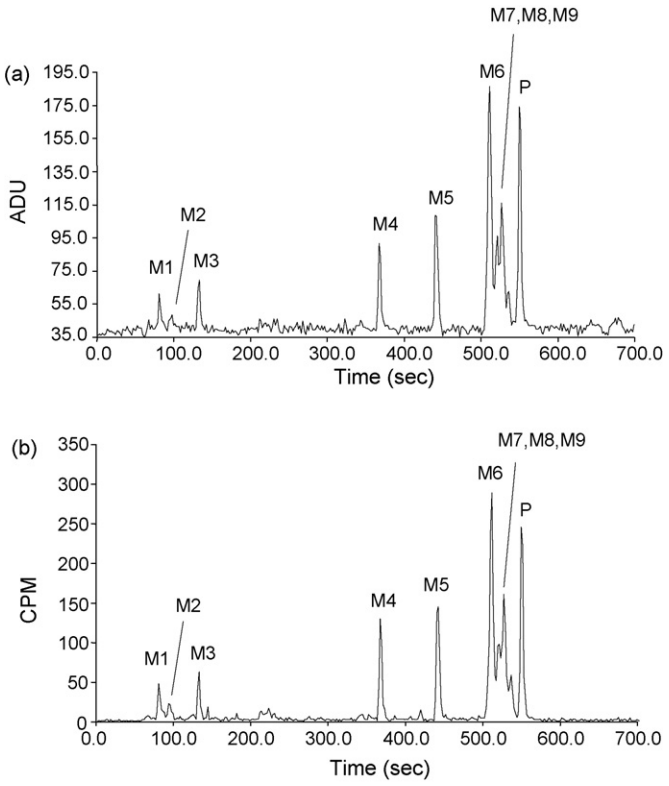

Fig. 4. Reconstructed radiochromatograms following UPLC of rat bile after administration of [^{14}C] drug-1 (in an isolated perfused rat liver model): (a) ViewLux and (b) TopCount. Fraction collection speed ~2 s/well, into Shallow-well LumaPlate-96 microplates.

3.3. Analysis of rat bile from a perfused rat liver ([^{14}C] drug-1)

Rat bile (0–4 h) from an isolated perfused rat liver study to investigate the metabolism of [^{14}C] drug-1, was used to assess the application of microplate imaging with real samples. Bile samples are probably the most complex biological matrix encountered in routine metabolism studies, and therefore represent a good examination for both chromatographic and radiochemical detec-

Table 2
ViewLux ADU inter- and intra-plate precision (Shallow-well LumaPlate-96)

	DPM											
	3	6	9	12	15	18	36	59	144	288	576	1151
Plate 1												
Mean[a]	42	51	59	69	78	88	142	218	479	919	1830	3638
STD	1.1	1.0	1.4	1.7	2.0	1.7	1.7	2.5	5.6	5.1	6.0	14.2
%CV	2.5	2.0	2.3	2.5	2.5	1.9	1.2	1.1	1.2	0.6	0.3	0.4
Plate 2												
Mean[a]	42	50	59	69	76	88	140	221	469	942	1815	3603
STD	1.0	1.0	0.9	1.3	1.1	1.9	1.7	1.8	3.8	3.8	10.2	15.3
%CV	2.3	2.0	1.6	1.9	1.5	2.2	1.2	0.8	0.8	0.4	0.6	0.4
Plate 3												
Mean[a]	41	50	59	69	77	86	138	217	468	911	1777	3505
STD	1.2	1.6	1.3	1.1	1.9	1.7	2.6	2.0	3.5	6.9	6.1	8.3
%CV	2.9	3.2	2.2	1.6	2.5	1.9	1.9	0.9	0.7	0.8	0.3	0.2
Plate 4												
Mean[a]	41	50	59	68	77	87	144	217	472	907	1787	3492
STD	1.0	1.1	1.7	1.2	1.7	2.6	2.8	4.4	4.4	5.9	7.3	10.0
%CV	2.5	2.2	2.8	1.7	2.3	2.9	2.0	2.0	0.9	0.7	0.4	0.3
Plate 5												
Mean[a]	40	51	58	68	77	88	140	217	478	924	1815	3577
STD	1.1	1.2	1.0	1.3	1.3	1.5	1.6	2.1	6.4	5.0	13.0	8.4
%CV	2.8	2.3	1.8	1.9	1.7	1.7	1.1	1.0	1.3	0.5	0.7	0.2
Between plate %CV	2.4	2.3	2.0	2.0	2.0	2.1	2.1	1.4	1.4	1.6	1.3	1.8

[a] $n = 10$.

tion methods. Robustness experiments conducted on UPLC coupled with TopCount using bile have been reported previously [4]. An aliquot of rat bile was injected onto the UPLC system as described in Section 2, and fractions collected into 3 × Shallow-well LumaPlate-96 microplates. Plates were dried and sealed and then counted or imaged using TopCount (40 min per microplate) or ViewLux (5 min per microplate), respectively. The metabolite radiochromatograms are compared in Fig. 4 and clearly show nine notable metabolites (assigned M1–M9) and unchanged parent drug (P). The metabolite profiles are quantitatively and qualitatively identical for both detectors.

3.4. Higher throughput (384-microplates)

Since the ViewLux and other CCD based systems can image entire microplates independent of plate well density, there are clearly further gains in throughput to be exploited by transferring to higher density microplates, such as 384 or 1536. In fact, the use of scintillant coated 384-well small volume microplates (e.g. Image FlashPlate®, PerkinElmer) is now routine in many non-separation HT screening assays, using a variety of isotopes [15–17]. The CTC HTX PAL post column fraction collector was used with a minimum collection time of approximately 2 s. Including the time taken for the fraction collector arm to move between wells, the total dwell time per fraction was ~2.3 s. At a flow rate of 300 μL/min approximately 10 μL per fraction is sequentially dispensed into each well (compatible with both 96- and 384-well microplate formats). The CTC HTX PAL was able to apportion fractions with the necessary precision requisite for 384-well microplates, dispensing approximately one droplet of eluent in the centre of each well. The *z*-axis of the CTC dispensing arm was adjusted, such that capillary action, when a droplet touched the base of the scintillant coated well, was sufficient to draw the eluent droplet off the dispensing needle, prior to the arm moving to the next well.

Microplates with even greater well density, such as 1536-well, are also being used in HTS applications, but are beyond the scope of this current fraction-collection equipment, in terms of dispensing arm precision. Furthermore, transferring methods to 1536-well microplates would require further miniaturisation of the chromatography. With optimised UPLC methods (as outlined above), 3–4 96-well microplates are adequate for fraction collection and subsequent off-line radiometric analysis on MSC systems, consistent with a shift to 384-well microplates. Based on current methodology one 384-well microplate would represent the entire chromatogram (*cf.* 3–4 96-well microplates) for most routine metabolite separations. This would bring further notable improvements to total exposure time (300 s per 384-well microplate, per radiochromatogram), and also potential benefits in up-front cost (less microplates) and subsequent disposal cost (less bulk radioactivity).

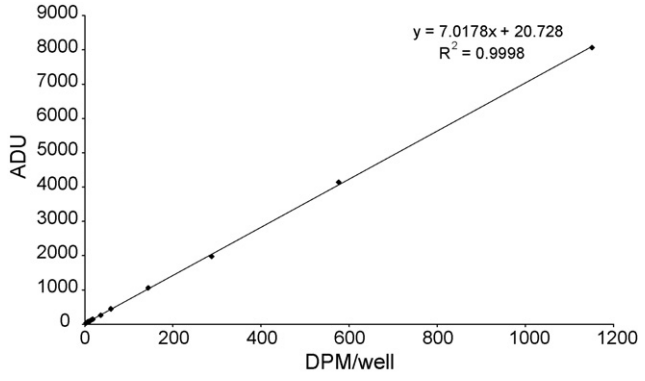

Fig. 5. A plot of ViewLux ADU response *vs.* DPM: LumaPlate-384 microplate [^{14}C] sucrose serial spiking ($n = 5$).

Table 3
ViewLux ADU *vs.* DPM (spiked LumaPlate-384 plates)

DPM	Background subtracted ADU[a]	STDEV	%CV
1151	8049	550	6.8
576	4123	67	1.6
288	1957	53	2.7
144	1038	55	5.2
59	428	8	1.7
36	239	15	5.8
18	127	6	4.3
15	104	6	5.1
12	76	6	5.9
9	61	3	3.3
6	41	3	5.6
3	14	2	5.0

[a] ADU $n = 5$ (background = 21 ± 2 ADU).

Three Deep Well LumaPlate®-384 microplates were spiked (15 μL) with increasing levels of [^{14}C]-sucrose: 3, 6, 9, 12, 15, 18, 36, 59, 144, 288, 576 and 1151 DPM ($n = 5$). A plot of the mean ViewLux ADU *vs.* the DPM spiked per well ($n = 5$) indicates that transitioning from 96- to 384-well formats does not impact the imager performance ($R^2 = 0.999$) over the radioactivity range tested (see Fig. 5). These data are tabulated in Table 3. Inter- and intra-plate precision was again assessed using five spiked LumaPlate-384 microplates (spiked as detailed above) imaged 10-times. These data demonstrated that the excellent robustness and reproducibility inherent in the LumaPlate-96 yttrium silicate design is not compromised by miniaturizing the well footprint from 96- to 384-well (data not shown).

The limit of detection and limit of quantification for the ViewLux using Deep Well LumaPlate-384microplates was calculated, to be approximately 1 and 3 DPM, respectively, using the standard approach outline above, and is two-fold improved compared to the 96-well microplate format. The greater sensitivity (*cf.* 96-well microplates) is, in part, due to more concentrated DPM (i.e. equal amount of DPM per fraction, but dried into a smaller microplate well area, producing more scintillant luminescence and hence more incident photo-electrons per pixel) and therefore higher signal-to-noise values.

It is established that an increase in MSC counting time can improve the counting sensitivity and signal-to-noise [13]. This is a feature which provides TopCount with a significant advantage over RFD methods. However, as stated elsewhere, increasing the count time will obviously increase the overall cycle time between sample

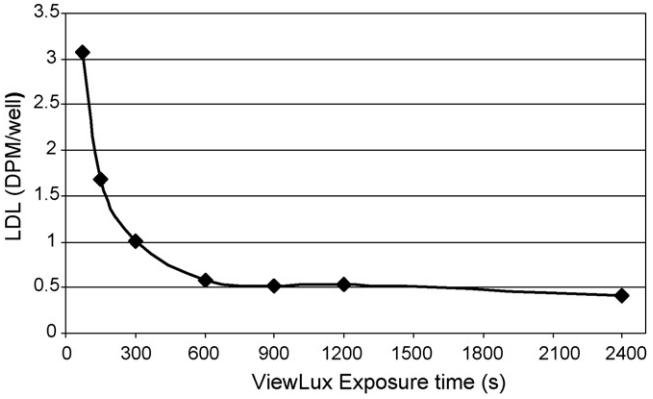

Fig. 6. A plot of lowest detection limit (DPM) of [^{14}C] sucrose in LumaPlate-384 microplate. *vs.* ViewLux measurement (exposure) times (75, 150, 300, 600, 900, 1200 and 2400 s).

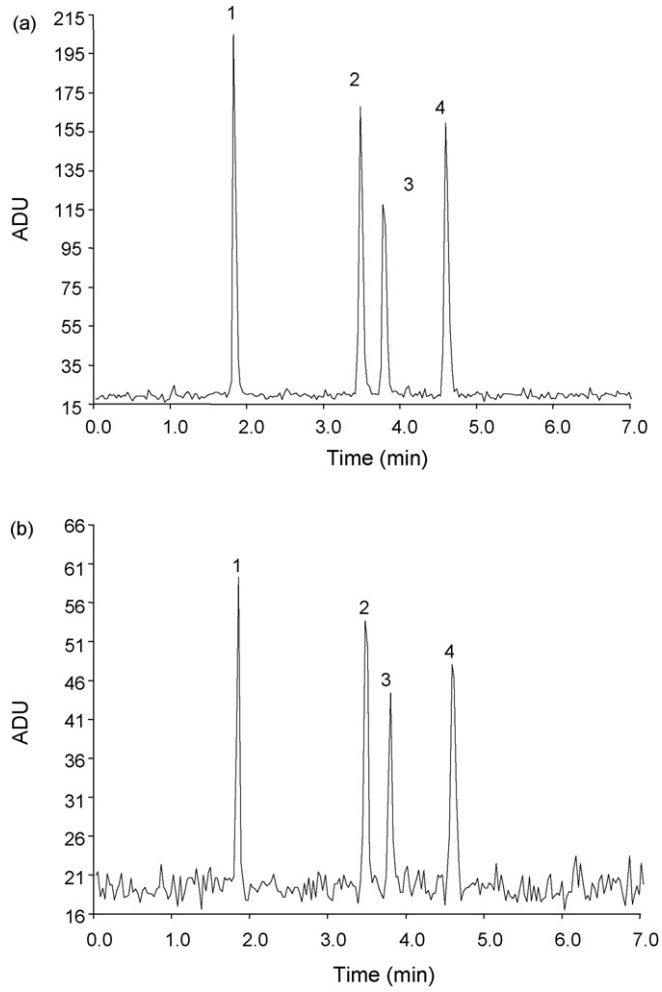

Fig. 7. ViewLux reconstructed radiochromatograms following UPLC of standard mix (LumaPlate-384 microplate): (a) 500DPM per analyte on-column and (b) 100DPM per analyte on-column (1 = caffeine, 2 = *S*-mephenytoin, 3 = chlorzoxazone, 4 = tolbutamide). Fraction collection speed ~2 s/well.

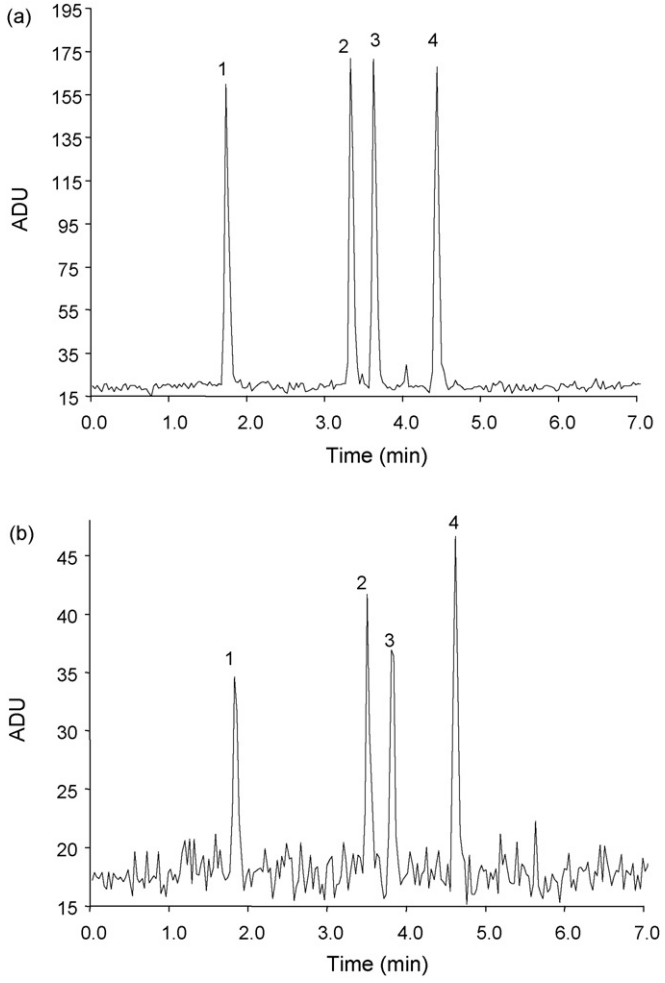

Fig. 8. ViewLux reconstructed radiochromatograms following UPLC of spiked human plasma extract (LumaPlate-384 microplate): (a) 500 DPM per analyte on-column and (b) 100DPM per analyte on-column (1 = caffeine, 2 = *S*-mephenytoin, 3 = chlorzoxazone, 4 = tolbutamide). Fraction collection speed ~2 s/well.

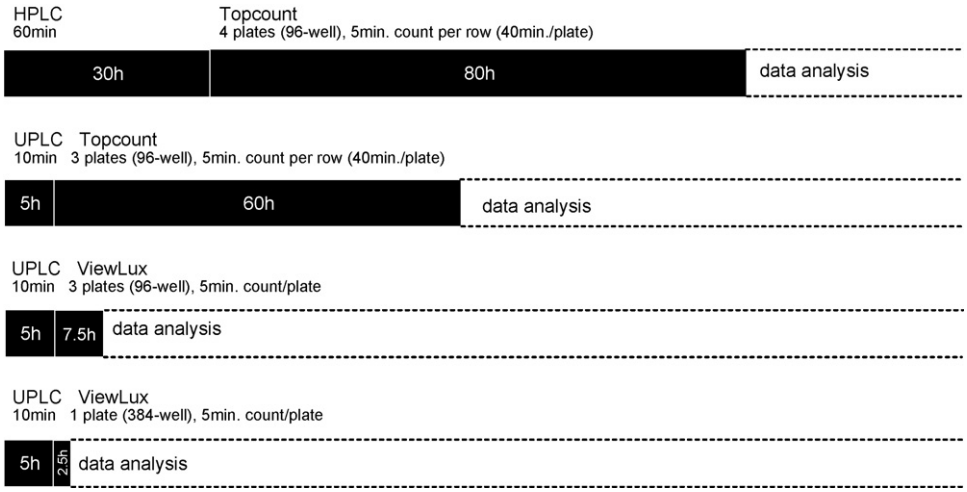

Fig. 9. Schematic of improvement in throughput transitioning from HPLC/TopCount to UPLC/ViewLux. The precise increase in total "study analysis" time, will actually depend on the length of the HPLC run time compared to the UPLC run time (arbitrarily set at 60 and 10 min, respectively in this case), the number of 96-well microplates used in fraction collection compared to the number of 384-well microplates plates used, and the respective count times of the detectors (arbitrarily set at 5 min per row for TopCount i.e. 40 min per microplate, and 5 min per microplate for ViewLux).

injection and data processing. In the case of ViewLux the count time (or image time) can be increased, for example 5–10 min, and still offer considerable throughput compared to TopCount. The imaging time is easily optimised to provide specified sensitivity for an assay. Lowest detection limit *vs.* imaging time for a [^{14}C]-sucrose spiked LumaPlate-384 microplate (see Fig. 6) indicates that the optimal range for the exposure time is 150–600 s.

The increased sensitivity is also evident in the reconstructed radiochromatograms generated using standard solutions and spiked plasma extracts. [^{14}C] caffeine, tolbutamide, chlorzoxazone and *S*-mephenytoin were again used to compare ViewLux response using 384-well microplates (Figs. 7 and 8) with TopCount using a traditional 96-well plate format, within a chromatography setting. Injection of standard mixtures and spiked human plasma (100 and 500 DPM per analyte) were made, as described previously. The signal-to-noise using 384-well format on ViewLux is now comparable to the signal-to-noise using a 96-well format on TopCount (Figs. 2 and 3 *vs.* Figs. 7 and 8). The image/count time for the ViewLux plate imaging was maintained at 10 min per microplate and for TopCount was 5 min per microplate row ($n = 12$ per row). This difference equates to a total count/image time of 120 min (3 × 96-well) using TopCount, and 10 min (1 × 384-well) using ViewLux. The superior throughput of image-based detection compared to PM-tube detection, is highlighted in Fig. 9. This figure serves to demonstrate the increase in throughput of the entire cycle time, by transferring from the worse case scenario (HPLC 60 min run time, with TopCount, 4 × 96-well microplate format) to the improved scenario, using current technology (UPLC 10 min run, with ViewLux, 1 × 384-well microplate format). The overall increase in throughput, across an entire "archetypal" metabolism study (with chromatographic and radiometric analysis of approximately 30 samples) is >10-fold. The increased throughput of UPLC *vs.* HPLC can thereby be matched by the increased throughput of the plate imager *vs.* the plate reader.

The 100 DPM spiked human plasma sample was used to assess the reproducibility of the entire methodology using UPLC separation and 384-well microplate collected fractions. The data are summarised in Table 4, following 10 replicate measurements of the respective chromatographic plate. For all analytes the ADU response demonstrates acceptable precision (CV 7% or less).

3.5. Analysis of rat bile from a perfused rat liver ([^{14}C] drug-2)

Rat bile samples collected following the administration of [^{14}C] drug-2 were used to assess the robustness of 384-well fraction collection and radioactivity measurement. [^{14}C] drug-2 rat bile samples were specifically chosen as these represented a very complex mixture of drug metabolites. An aliquot of rat bile was injected onto the UPLC system as described in Section 2, and fractions collected into either 4 × Shallow-well LumaPlate-96 microplates or a single Deep Well LumaPlate-384 microplate. The Shallow-well LumaPlate-96 microplates were dried and then counted using TopCount (2 min per microplate row, 64 min in total). The LumaPlate-384 microplate was also dried and then imaged using ViewLux (5 min in total). The metabolite radiochromatograms are compared in Fig. 10 and clearly show five notable metabolites (assigned M1–M5) and unchanged parent drug (P), together with a complex mixture of other minor metabolites. The metabolite profiles of the major components are comparable for both detectors.

Table 4
ViewLux ADU precision for UPLC chromatographed analytes (LumaPlate-384 microplates)

Run #	ADU[a]			
	Caffeine	Mephenytoin	Chlorzoxazone	Tolbutamide
1	38	50	50	59
2	42	44	47	52
3	42	52	48	53
4	42	53	44	51
5	39	47	53	54
6	41	51	49	53
7	41	48	45	55
8	39	54	46	53
9	43	49	47	48
10	42	55	46	56
Mean	40.9	50.3	47.5	53.4
STD	1.7	3.4	2.6	3.0
CV%	4.1	6.8	5.5	5.5

[a] ADU at chromatographic peak apex.

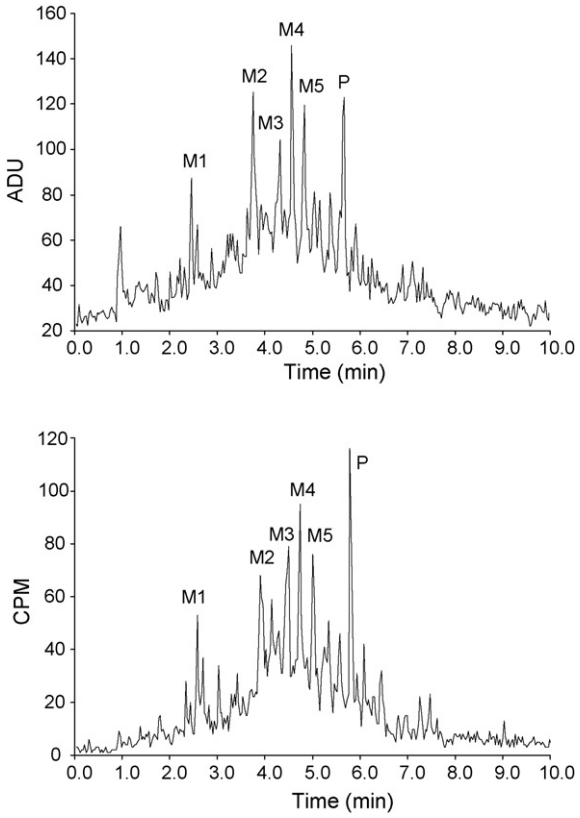

Fig. 10. Reconstructed radiochromatograms following UPLC of rat bile after administration of [^{14}C] drug-2 (in an isolated perfused rat liver model): (a) ViewLux and (b) TopCount. Fraction collection speed ~2 s/well, into LumaPlate-384 microplate or Shallow-well LumaPlate-96 microplates.

3.6. Future developments

Since CCD chips are most sensitive to light emitted in the red region of the spectrum (>600 nm), and LumaPlate® microplates use standard yttrium silicate emitting in the blue-region of the spectrum (emission wavelength for standard yttrium silicate 390–420 nm), further sensitivity gains may be possible by using modified scintillant formats. In addition the majority of compounds that cause colour quenching are yellow or brown in colour, and therefore absorb light in the blue region of the spectrum. Therefore, quenching effects should be less obvious with red-emitting scintillant.

4. Conclusion

Microplate imaging, in conjunction with UPLC separation, offers an alternative approach for the detection and quantification of drug metabolites in complex biological samples. The ViewLux is able to count (image) plates with increased throughput when using a standard 96-well microplate format, compared to MSC systems, providing data in a quicker timeframe. This can be further enhanced by transferring to 384-well formats, which enable a chromatogram to be collected into a single microplate *in toto*. Since the ViewLux can image independently of plate well density, this produces a significant boost in the ability to evaluate data at the time of separation. The ViewLux, offers all the advantages of more traditional PM-tube based plate readers (such as sensitivity *cf.* RFDs), but without compromising the cycle time, i.e. the time it takes to count collected fractions for radioactivity is similar to the time it takes to chromatograph sample using UPLC methodology. The sensitivity and precision of the ViewLux for detecting [^{14}C]-drug metabolites in isolated biological fractions is comparable to that of a high end PM-tube based plate reader such as TopCount. The combination of UPLC, ViewLux and Deep Well LumaPlate-384 thereby provides an extremely sensitive, higher throughput platform for separating, detecting and quantifying drug metabolites.

Acknowledgments

The authors would like to thank Sebastian Pearce, Frederic Bernard, Lisa Osborne and Martin Pluthero for generating samples from the isolated perfused rat model, and Rob van Beurden and Alan Fisher for providing samples of the new Deep Well LumaPlate-384 microplates.

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