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TopCount coupled to ultra-performance liquid chromatography for the profiling of radiolabeled drug metabolites in complex biological samples

G.J. Dear*, N. Patel, P.J. Kelly, L. Webber, M. Yung

DMPK, GlaxoSmithKline R&D, Ware, Herts. SG12 0DP, UK

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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 (UPLC). It has recently been shown that 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 TopCount, a microplate scintillation counter, to act as a suitable radiodetection system for ultra-performance liquid chromatography methods is tested. TopCount, has innumerable benefits over more traditional on-line radioactivity flow detection methods, when dealing with the narrow peak widths and small peak volumes associated with the enhanced efficiency of sub-2 μm columns. The system is tested for robustness and sensitivity, and then used to undertake successful metabolite profiling of actual samples, and the data compared to traditional HPLC with on-line radioactivity flow detector.

Keywords: Ultra-performance liquid chromatography; Metabolites; Radiochemical detection; TopCount

1. Introduction

The identification of drug metabolites is an established part of the drug development process, and is required to validate the toxicology species in support of safety testing, and in particular provides information on the potential to form pharmacologically active metabolites, or metabolites with toxicological consequences in man. Traditionally radiolabelled drug candidates are used in these metabolism studies, to act as a tracer to help identify and isolate (if necessary) drug metabolites, but more importantly the use of radiolabelled drugs is essential to facilitate the quantification of drug and metabolites in complex samples such as excreta. High performance liquid chromatography (HPLC) has become the analytical method of choice, for separating drug metabolites in biological samples (in vitro and in vivo), and is routinely coupled to on-line radioactivity flow detectors (RFD), to enable facile detection and quantification of drug and metabolites [1,2]. RFDs can also be coupled to liquid chromatography/mass spectrometry (LC/MS), mediated through HPLC compatible atmospheric pressure ionisation (API) sources, such as electrospray [3]. However, RFDs can lack sensitivity, compared to static liquid scintillation counting (LSC) [4], and microplate scintillation counters (MSC), such as TopCount, have been developed as an off-line radiodetector, using automated fraction collection of HPLC eluent [4–9]. The key benefits of these systems over traditional LSC include the use of low cost solid scintillant plates and multiple arrays of photomultipliers which greatly reduce the overall effort and counting time. The validation of MSC as a high sensitivity HPLC radiodetector has recently been the subject of a comprehensive review [10]. MSC systems are also compatible with the low flow rates and small peak volumes associated with miniaturised separation techniques such as capillary HPLC and capillary electrophoresis [11].

Although HPLC has become the linchpin of drug metabolite profiling and identification studies, the recent introduction of rugged small particle packed columns (<2 μm), and the commercialisation of instrumentation which can withstand the ensuing high pressures, has lead to an increase in the application of so-called ultra-performance liquid chromatography (UPLC) in this field [12–14]. It is well documented that a decrease in particle size results in numerous benefits to the chromatographer, includ-

* Corresponding author. Tel.: +44 1920 88 4285; fax: +44 1920 88 4215.
E-mail address: gordon.j.dear@gsk.com (G.J. Dear).

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ing a significant gain in efficiency, an increase in sensitivity and a concomitant increase in peak capacity per unit time in gradient separation [15]. Furthermore, the low system and gradient dwell volumes of UPLC instruments provide the opportunity to fully exploit the advantages of sub-2 μm particle technology. Many reports have recently been published detailing the advantages of UPLC/MS for metabolite identification [12–14]. However, if UPLC is to truly compete with HPLC as a separation technique for the analysis of drug metabolites in complex matrices, then a suitable means of radiodetection is required, together with a full assessment of the robustness of UPLC to biological samples typically encountered in drug metabolism studies (e.g. excreta and tissue extracts).

The use of RFDs with UPLC is severely limited, due to the large volume of the homogeneous liquid cells routinely employed (150–1000 μL). The post-column band broadening caused by such detectors, is extremely prohibitive, when dealing with UPLC peaks in the range of 2–6 s in width, which equate to peak volumes of approximately 10–20 μL. Smaller cells are available (e.g. 40 μL) but these are still large compared to the eluting peak volumes, and can limit chromatographic performance when using UPLC. Decreasing the residence time in the cell by increasing the scintillation flow rate, can compensate to some degree, by limiting the potential for peak dispersion, but will significantly reduce sensitivity. In this paper MSC is applied as an alternative radiodetector for UPLC, in combination with a modified rapid fraction collection system. The system has been tested for robustness to biological samples, and the limits of detection established, and then applied to the separation of drug metabolites, in dog plasma following the administration of a novel drug candidate. The results obtained by UPLC with TopCount are compared here to traditional HPLC coupled to a RFD and TopCount.

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] drug-1 was 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) was supplied by Sigma–Aldrich Co. (Dorset, UK). Control dog urine, bile, plasma and faeces were obtained from GlaxoSmithKline Research and Development, Ware, 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 models LS6000IC, Beckman Coulter UK Limited, High Wycombe, Bucks., UK), following the addition of 10 mL of Startscint scintillation fluid (Packard Limited, Pangbourne, Berks, UK). This confirmed that the radioactive concentration was approximately 8000 dpm per analyte, and this data was used to determine the counting efficiency of both the UPLC/TopCount and HPLC/RFD systems. This stock solution was also used for spiking all samples as detailed below. Additional dilutions were made in buffer, where appropriate, to conduct the linearity testing.

2.3. Preparation of spiked samples

Spiked samples used for robustness testing were generated by spiking known amounts of the standard solutions into control rat urine, bile, plasma extract and faecal extract to produce final concentrations of 8 dpm/μL for each analyte (16 dpm/μL for spiked bile). Plasma and faecal extractions were conducted as described below.

2.4. Sample extraction

Control rat 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).

A control rat faecal homogenate was extracted by vortex-mixing aliquots (2 g) 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. Each sample was then centrifuged at approximately 1500 × *g* at 4 °C for 10 min. The supernatants were 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).

Both plasma extracts and faecal extracts were filtered through 0.45 μm polypropylene filter units (Mini-Uniprep™, Whatman, NJ, USA).

2.5. Dog plasma following administration of drug-1

Pooled plasma obtained 1 h following oral administration of [^{14}C] drug-1 to three Beagle dogs, at a nominal dose of 5 mg/kg (in 0.5% (w/v) aqueous hydroxypropylmethyl cellulose containing 0.1% (w/v) Tween 80) was extracted by vortex-mixing aliquots (1 mL) for approximately 1 min with four 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 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 2.5 mL water/acetonitrile (v/v, 50/50).

To determine the counting performance of the UPLC/TopCount and HPLC/RFD systems the level of radioactivity in pooled dog plasma extracts (20 μL) was determined by liquid scintillation counting (Beckman models LS6000IC, Beckman Coulter UK Limited, High Wycombe, Bucks., UK), following the addition of 10 mL of Startscint scintillation fluid (Perkin-Elmer LAS (UK) Limited, Beaconsfield, Bucks., UK).

2.6. UPLC

All UPLC separations were performed on a Waters Acquity UPLC™ system (Waters, Manchester, UK), with an upper pressure limit of ca. 1000 bar [16,17], and equipped with a binary pump, an autosampler and a tunable UV detector. Injections of samples 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, pH 5 (A) and methanol (B). For all robustness and validation experiments the proportion of methanol 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 the methanol 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®, Perkin-Elmer LAS (UK) Limited, Beaconsfield, Bucks., UK) 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 (v4.0) software (Waters, Manchester, UK).

For the UPLC analysis of dog plasma extracts obtained following administration of [^{14}C] drug-1, separations were performed with a mobile phase of 50 mM ammonium acetate, pH 4.5 (A) and acetonitrile (B). The proportion of acetonitrile was programmed to linearly increase from 10 to 60% in 11.6 min, with a further increase to 95% at 15 min. The proportion of acetonitrile was then held at 95% until 16.5 min and then returned to 10% acetonitrile. The column was then allowed to re-equilibrate for approximately 2 min prior to the next injection. All other conditions are as described above.

2.7. HPLC

HPLC analysis, with on-line RFD, of dog plasma extracts following administration of [^{14}C] drug-1 was conducted on an Agilent model 1100 gradient liquid chromatograph (Agilent, Waldbronn, Germany), with an upper pressure limit of ca. 400 bar. Separations were achieved on a 250 mm × 4.6 mm i.d., 5 μm, Hypersil GOLD column (Thermo Hypersil-Keystone, Cheshire, UK) operated at 30 °C, using a mobile phase of 50 mM ammonium acetate, pH5 (A) and acetonitrile (B). The proportion of acetonitrile was programmed to linearly increase from 10 to 60% over 35 min, with a further increase to 95% at 45 min. The proportion of acetonitrile was then held at 95% for 5 min. The column was then allowed to re-equilibrate at 10% acetonitrile prior to the next injection. All analyses were performed at a flow rate of 1 mL/min. The radioactivity signal was monitored using a Lablogic ß-RAM radioactivity detector (Lablogic Systems Ltd., UK), with a 500 μL liquid cell and continuous mixing of the eluent with 3 mL/min Flowlogic Maxcount scintillation fluid (Lablogic Systems Ltd., UK). Counting time was approximately 7.5 s. Instrument control and data processing were performed on Laura software, version 3.3 (Lablogic Systems Ltd, UK).

HPLC analysis, with off-line fraction collection, was conducted on a Merck Hitachi L6200A HPLC system (Merck, Darmstadt, Germany), with an upper pressure limit of ca. 400 bar. Separations were achieved on a 250 mm × 4.6 mm i.d., 5 μm, ODS2 Prodigy column (Phenomenex, Cheshire, UK) operated at 40 °C, using a mobile phase of 50 mM ammonium acetate, pH5 (A) and methanol (B). The proportion of methanol was maintained at 10% for 5 min and then programmed to linearly increase from 10 to 90% over 25 min, and then held at 90% for a further 8 min. The column was then allowed to re-equilibrate for 10 min prior to the next injection. All analyses were performed at a flow rate of 1 mL/min. HPLC fractions were collected into 96-well microplates (Deep-Well LumaPlate®, Perkin-Elmer LAS (UK) Limited, Beaconsfield, Bucks., UK) at a rate of ∼9 s/well using a Gilson XL-222 liquid handler (Gilson, Villiers le Bel, France).

2.8. TopCount

After collection all LumaPlate® microplates were dried in an oven at 60 °C and then sealed using microplate heat sealing film (TopSeal-S, Perkin-Elmer LAS (UK) Limited, Beaconsfield, Bucks., UK). Radioactivity (CPM value) of the dried residues in the 96-well plates was determined using a TopCount NXT (Perkin-Elmer LAS (UK) Limited, Beaconsfield, Bucks., UK), 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.

3. Results and discussion

3.1. System set-up

The success of high efficiency separations achieved with small particle packed columns (<2 μm) is dependent, in-part, on the system configuration. The Acquity UPLC™ instrument used in this work is designed with a very small system volume (∼120 μL), such that any peak dispersion not associated with the chromatographic separation is minimised [16,17]. In order to maintain this chromatographic performance the dead volume post-column needs to be kept to a minimum. To this end the Acquity and the CTC HTX PAL fraction collector were

optimally positioned in order to reduce the length of transfer tubing required. A single piece of PEEK 65 μm internal diameter tubing was used to connect the column exit with the fraction collector head. The total volume post-column was <0.5 μL. The CTC HTX PAL was fitted with an upgradeable micro collection/spotting option to provide a fast and effective means of transferring low volume fractions into the 96-well LumaPlate® solid scintillant plates.

The next consideration was the collection time of the fraction collector. To maintain chromatographic resolution the fraction collection volume should ideally be less than the peak volume. Most HPLC applications with TopCount rely on a fraction collection time of 7–15 s, which is consistent with the peak widths and peak volumes commonly encountered in these methods [4]. The extra chromatographic efficiency of UPLC routinely produces peak widths of 3–6 s. In this work with UPLC the CTC HTX PAL 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 this would equate to approximately 10 μL per fraction. Based on UV data from the integrated Acquity TUV (cell volume 0.5 μL), peak widths for standard solutions of caffeine, tolbutamide, *S*-mephenytoin and chlorzoxazone were ca. 3 s. Using a flow rate of 300 μL/min on a 2.1 mm i.d. column this corresponds to peak volumes in the region of 15 μL. These volumes are compatible with the fraction collection regime employed.

A representative UPLC radiochromatogram of a standard mix containing caffeine, tolbutamide, *S*-mephenytoin and chlorzoxazone is shown in Fig. 1, with each analyte representing approximately 125 dpm on column. All four peaks were readily separated in less than 10 min, using a simply linear gradient. Since the smaller particle packed column imparts extra chromatographic efficiency the analysis time can be reduced using shorter columns (10 cm in this case) without any loss in resolution [12,15]. This combined with the narrower peaks and increased peak capacity per unit time associated with UPLC, gives rise to the superior throughput compared to conventional

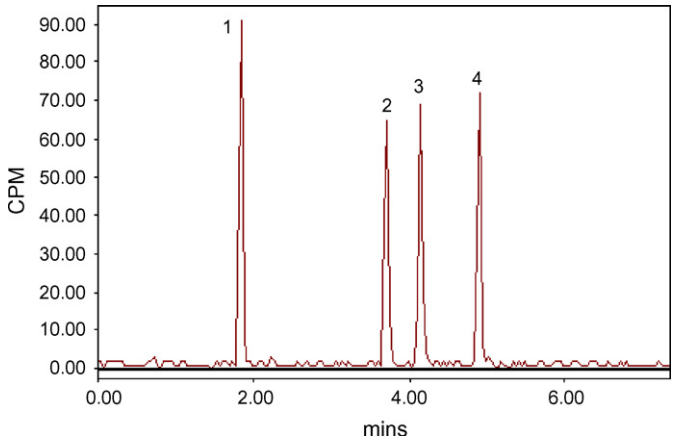

Fig. 1. UPLC/TopCount radiochromatogram of the standard mixture (1, caffeine; 2, *S*-mephenytoin; 3, chlorzoxazone; 4, tolbutamide, ca. 125 dpm per analyte, on-column). Fraction collection speed ~2 s/well. 5 min counting time.

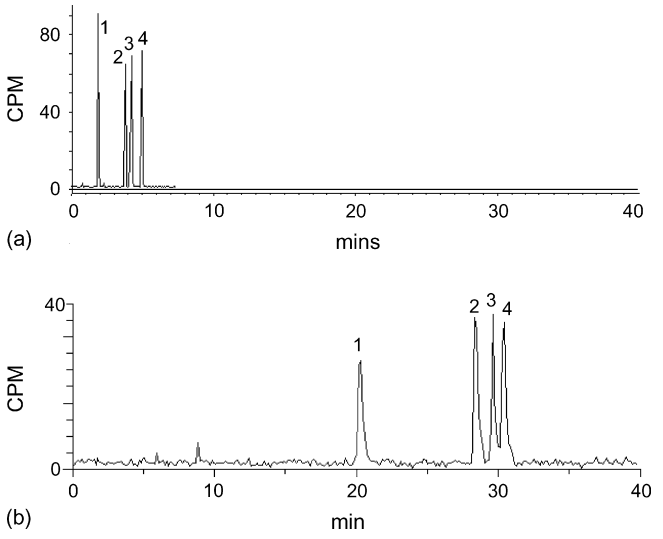

Fig. 2. (a) UPLC/TopCount radiochromatogram of the standard mixture (1, caffeine; 2, *S*-mephenytoin; 3, chlorzoxazone; 4, tolbutamide, ca. 125 dpm per analyte, on-column). Fraction collection speed ~2 s/well. 5 min counting time. (b) HPLC/TopCount radiochromatogram of standard mixture. Fraction collection speed ~9 s/well. 5 min counting time.

HPLC, as testified by this 5 min separation. This is evident by comparison of the UPLC/TopCount radiochromatogram with an equivalent HPLC/TopCount radiochromatogram (see Fig. 2). Both chromatograms represent 125 dpm on column (per analyte), but the UPLC radiochromatogram displays increased peak intensity and a superior signal-to-noise ratio. Peak widths in the UPLC radiochromatogram were ≤10 s. Examination of the raw ASCII file from each UPLC TopCount chromatogram revealed that the majority of the radioactivity for each analyte was contained within 2–3 wells, thereby maximising the dpm collected per fraction, while maintaining the chromatographic resolution.

Due to the low flow rate and the speed of fraction collection, the *z*-axis of the CTC collection arm was adjusted so that drops exiting the fraction collection needle touched the bottom of the wells in the LumaPlate® solid scintillant plates. This served to improve the accuracy of fraction collection, with approximately equal volumes dispensed per well, with no fractions missed. A flow rate of 300 μL/min was used for all analyses, since at higher flow rates the fraction collector speed was not sufficient, causing fraction collection to become erratic.

3.2. Validation

The validation and application of TopCount off-line radiodetection for HPLC analysis of drug metabolism samples has been the subject of many previous publications [4–9]. In particular the sensitivity, accuracy, precision and matrix effects incurred by this approach have been extensively reported [10]. Therefore, it is not the intention in this paper to re-address validation of this detection technique. However, since to the authors' knowledge, this represents the first application of UPLC coupled to TopCount, some rudimentary assessment of performance was ascertained, in the unlikely event that the use of UPLC instead of HPLC had compromised the validity of the TopCount method.

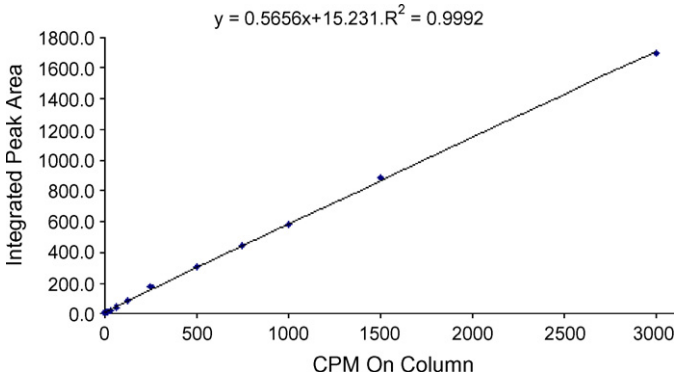

Fig. 3. A plot of integrated peak area response vs. dpm on column for UPLC/TopCount system (analyte = tolbutamide). Fraction collection speed ~2 s/well. 5 min counting time.

Using standard solutions of tolbutamide the linearity of the UPLC/TopCount system was assessed, by injecting increasing volumes of solution, corresponding to approximately 5, 15, 30, 62.5, 125, 250, 500, 750, 1000, 1500 and 3000 dpm on column. Tolbutamide was chosen, since this was the last eluting peak of the selected standards and therefore was the analyte most likely to be effected by peak dispersion during separation. A plot of the peak area response versus the dpm on column indicates that TopCount coupled with UPLC separation provides a linear response across the radioactivity range tested, $R^2 = 0.999$ (see Fig. 3). A radiochromatogram of the standard solution, corresponding to 62.5 dpm on column is shown in Fig. 4.

The limit of detection for UPLC/TopCount, using a 5 min counting time, 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). The blank proportion of the radiochromatogram between 0 and 1 min was used to determine the background CPM ($n = 11$). This provides a limit of detection for UPLC/TopCount of approximately four CPM, which is consistent with previously published values for HPLC/TopCount [4,10]. 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 seven CPM using this system, based on accepted rational, $y = y_B + 10s_B$. Again this value is consistent with previously reported values [4,10].

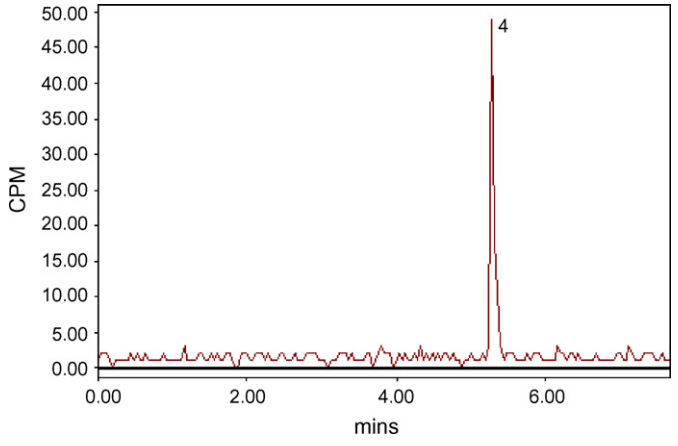

Fig. 4. UPLC/TopCount radiochromatogram of standard solution (4, tolbutamide, ca. 62.5 dpm on-column). Fraction collection speed ~2 s/well. 5 min counting time.

It has also been reported that an increase in counting time can improve the counting sensitivity [4]. This is a feature which provides TopCount with a significant advantage over RFD methods, where count times are limited, as determined by the residence time in the homogeneous radio cell (for typical RFD methods the radioactive peak is counted for 5–15 s). The ability of combining longer count times with the narrow peak widths of UPLC provides a system with enhanced sensitivity, which will undoubtedly be useful for occasions where dose level is limited by toxicology or route (e.g. inhalation) or where sample availability is limited (e.g. mouse plasma).

3.3. Robustness

The detection, quantification and identification of drug metabolites is inherently reliant on the successful analysis of complex samples which range from crude excreta extracts to relatively simpler in vitro preparations. Following initial assessment of UPLC/TopCount performance, the robustness of the system was evaluated using spiked samples. A typical rodent or non-rodent metabolism study in our laboratory involves the analysis of ca. 50 samples, which includes injection of spiked controls for stability assessment, column recovery experiments and actual study samples. It therefore seems reasonable that the UPLC/TopCount system described here should offer robust chromatography in terms of resolution and retention time reproducibility over an entire study without the need to substitute the column (i.e. ca. 50 injections of matrix). For this evaluation four new columns were tested by repeat injections of spiked controls: bile, urine, faecal extract and plasma extract, containing approximately 8 dpm/μL for each spiked analyte (except bile ~16 dpm/μL). These served to mimic typical study samples. Each new column was reserved for a single matrix.

Where possible the maximum injection volume (20 μL) afforded by the autosampler configuration was used. However, due to the high levels of organic solvent in plasma and faecal extracts, the injection volume was purposely limited to 10 μL when analysing these matrices, to help maintain the chromatography. A representative radiochromatogram of spiked faecal extract, following the injection of 20 μL, is shown in Fig. 5, and revealed notable distortion of the early eluting caffeine peak, caused by the methanol present in the injected sample. Radiochromatograms of rat faecal extract and bile, corresponding to the 1st and 50th injection are shown in Fig. 6, and demonstrate that the system as tested is robust to the typical samples encountered in a drug metabolism studies. Comparable data for spiked rat plasma and rat urine are shown in Fig. 7.

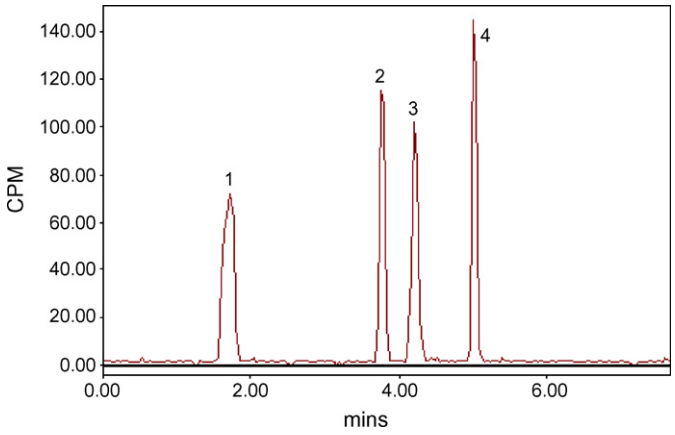

Fig. 5. UPLC/TopCount radiochromatogram of spiked rat faecal extract (1, caffeine; 2, *S*-mephenytoin; 3, chlorzoxazone; 4, tolbutamide, ca. 160 dpm per analyte on-column, 20 μL injection). Fraction collection speed ~2 s/well. 5 min counting time.

3.4. Analysis of dog plasma following the administration of drug-1

To test the combined UPLC and TopCount method with real samples, plasma extracts originating from a dog metabolism study following the oral administration of [^{14}C] drug-1 were analysed, as described in the experimental. A radiochromatogram corresponding to a pooled 1 h plasma extract is shown in Fig. 8, corresponding to a 5 μL injection of extract. The on-column level of radioactivity was equivalent to approximately 3000 dpm, which equates to 8 ng equivalents [^{14}C] drug-1 related material. Four peaks (parent drug and three metabolites) are clearly separated in less than 15 min. A comparable radiochromatogram obtained using a previously developed HPLC method, coupled with an on-line RFD is also shown in Fig. 8, corresponding to a 50 μL injection. The on-column level of radioactivity was equivalent to approximately 30,000 dpm, but nevertheless the signal-to-noise ratio was essentially the same in both radiochromatograms. In addition, although the analysis time has been significantly decreased, the resolution between the metabolites and parent drug is approximately the same using both methods. The decrease in chromatographic run time will increase the throughput for these types of analyses and may also reduce method development time. The counting efficiency of each method was assessed by comparing the total counts recorded using Laura software peak integration following injection of the standard stock solution (ca. 8000 dpm per analyte) with the total sample counts as determined by liquid scintillation counting. The counting efficiency was determined

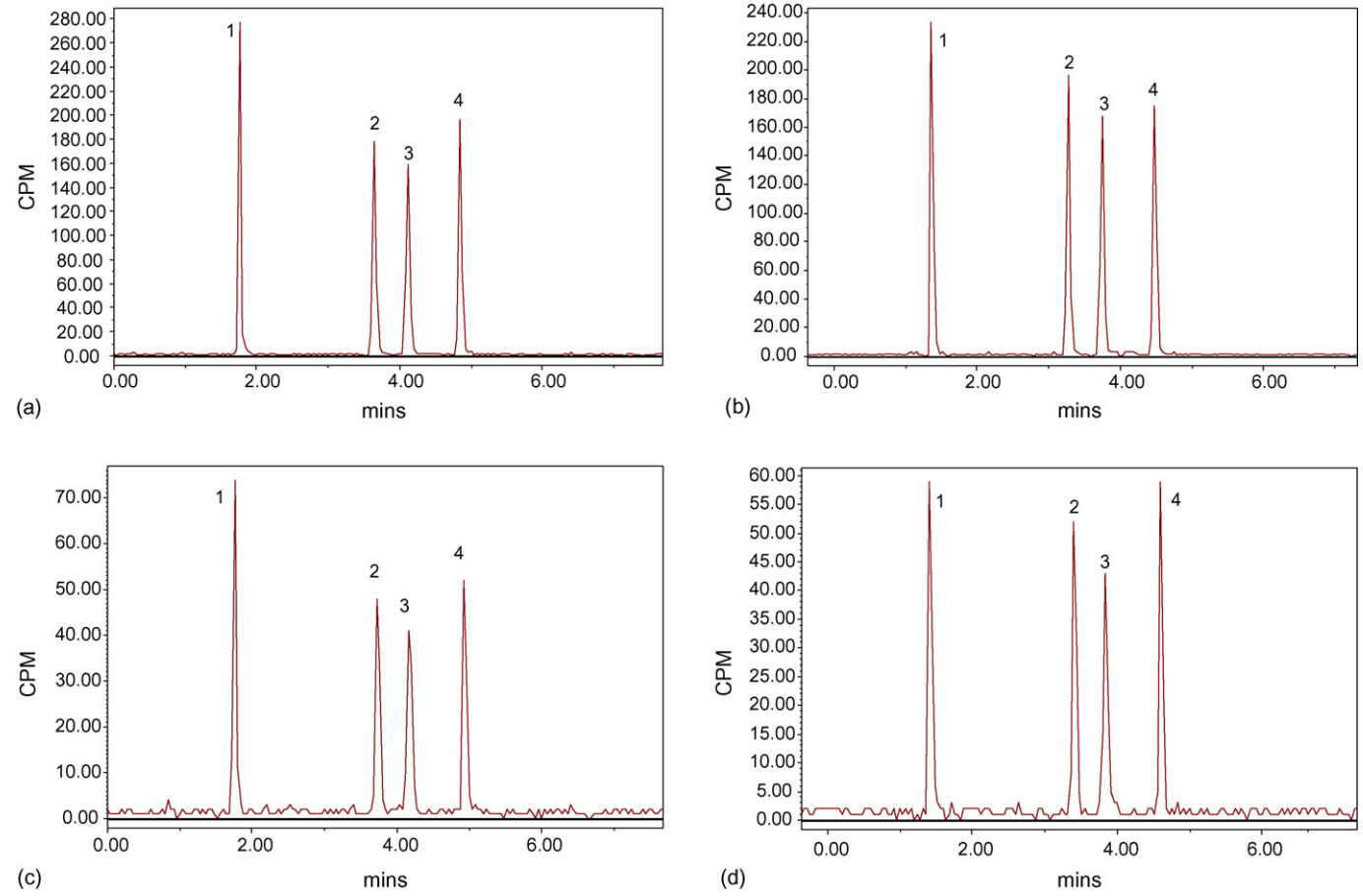

Fig. 6. UPLC/TopCount radiochromatograms of spiked rat bile (a) 1st injection and (b) 50th injection, and spiked rat faecal extract (c) 1st injection and (d) 50th injection (1, caffeine; 2, *S*-mephenytoin; 3, chlorzoxazone; 4, tolbutamide, ca. 160 dpm per analyte on-column, 20 μL injection for bile and ca. 80 dpm per analyte on-column, 10 μL injection). Fraction collection speed ~2 s/well. 5 min counting time.

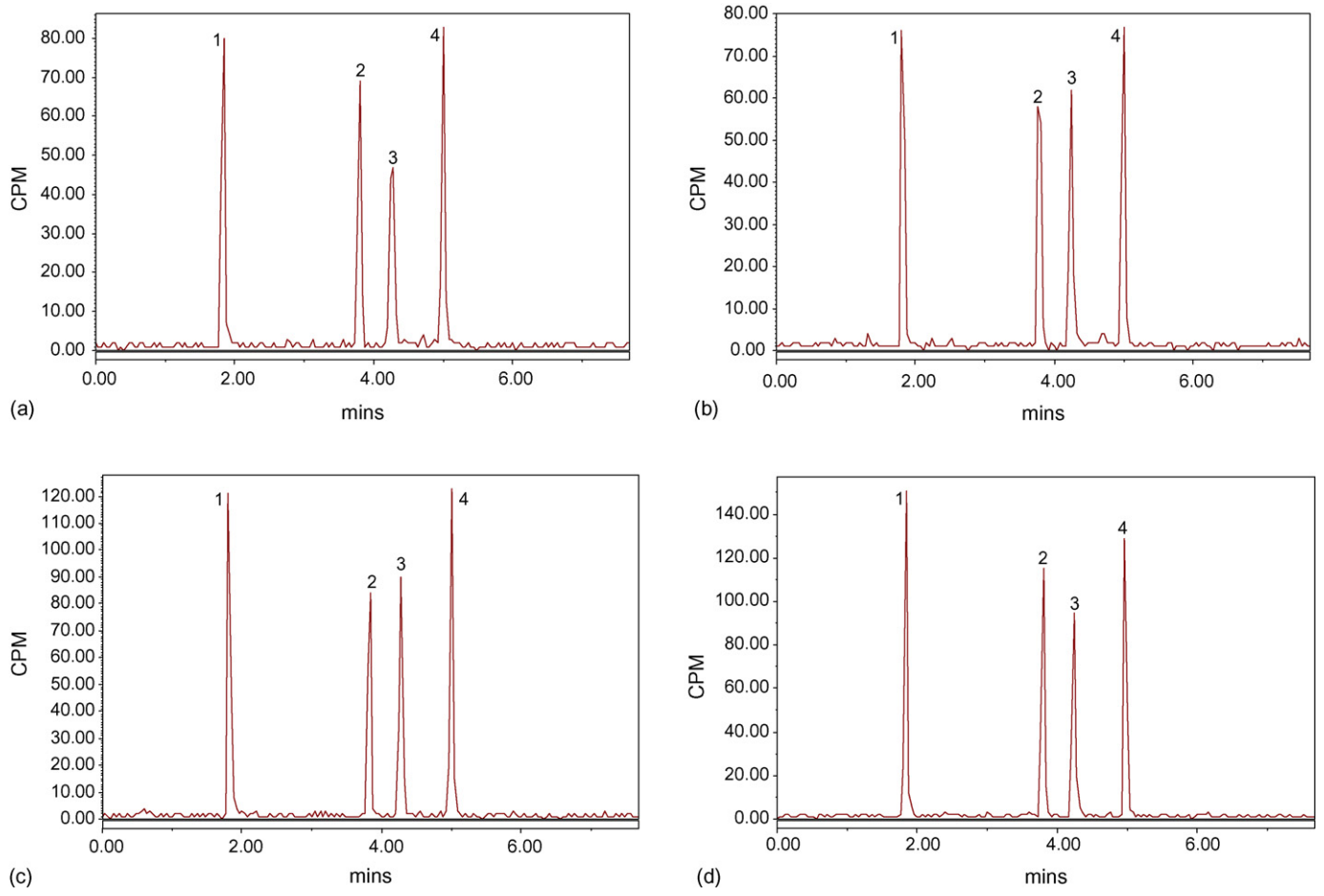

Fig. 7. UPLC/TopCount radiochromatograms of spiked rat plasma (a) 1st injection and (b) 50th injection, and spiked rat urine (c) 1st injection and (d) 50th injection (1, caffeine; 2, *S*-mephenytoin; 3, chlorzoxazone; 4, tolbutamide, ca. 160 dpm per analyte on-column, 20 μL injection). Fraction collection speed ~2 s/well. 5 min counting time.

to be ~80 and ~90% for HPLC/RFD and UPLC/TopCount systems, respectively.

Dog plasma extract was further diluted with mobile phase buffer and then re-injected producing a radiochromatogram corresponding to ca. 150 dpm on-column (see Fig. 9). Metabolites 2 and 3 represented approximately 5–7 CPM and are therefore on the limit of detection for the system. However, despite the low levels of radioactivity injected, the metabolite profile is still readily discernible demonstrating that UPLC combined with TopCount is an ideally suited system for analysing low levels

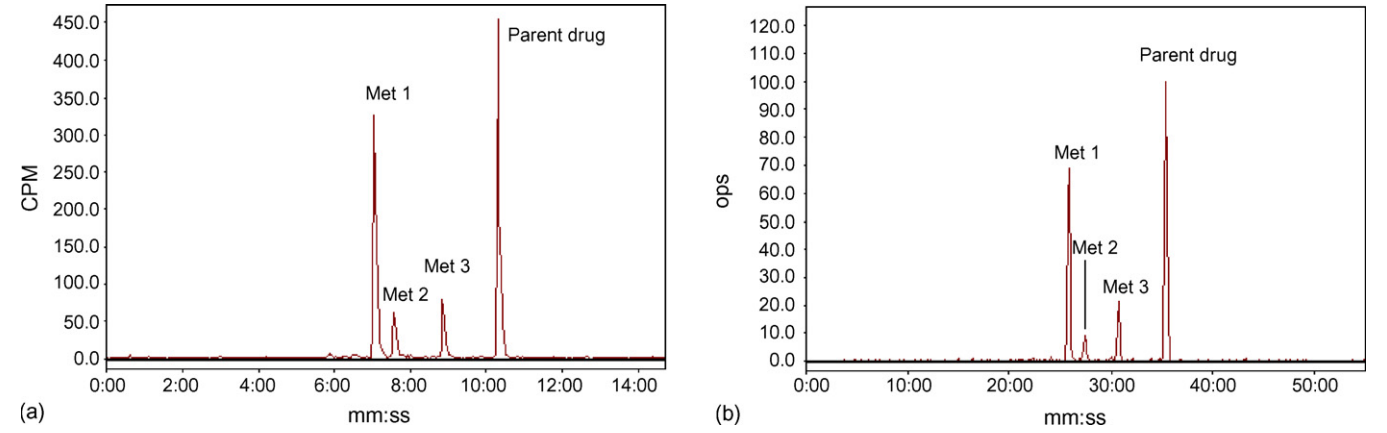

Fig. 8. (a) UPLC/TopCount radiochromatogram of dog plasma (1 h) following oral administration of [^{14}C] drug-1 (ca. 3000 dpm on-column). Fraction collection speed ~2 s/well. 5 min counting time. (b) HPLC/RFD radiochromatogram of dog plasma (1 h), following oral administration of [^{14}C] drug-1 (ca. 30,000 dpm on-column).

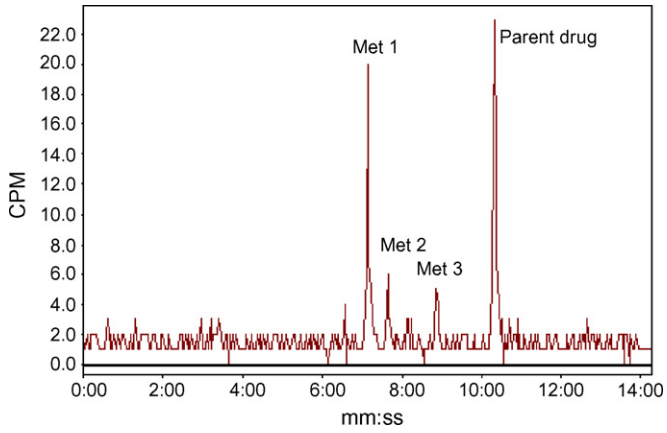

Fig. 9. UPLC/TopCount radiochromatogram of dog plasma (1 h) following oral administration of [^{14}C] drug-1 (ca. 150 dpm on-column). Fraction collection speed ~2 s/well. 5 min counting time.

of radioactive metabolites, in biological samples. The enhanced efficiency of UPLC and the reduced drying times of low volume fractions, increases the throughput of the methodology, to culminate in a faster, more sensitive approach compared to traditional HPLC/RFD methods.

4. Conclusion

The combination of UPLC and microplate scintillation counting (TopCount) offers both high sensitivity and high resolution separations. Using fraction collection and an off-line scintillation counting mechanism, the separation and detection can become uncoupled, such that the speed and configuration of the fraction collection process can be optimised to meet the needs of UPLC. Such flexibility is currently not available from 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. Importantly UPLC was found to offer robust and reproducible chromatography, even when subjected to repeat injections of crude samples typically associated with drug metabolism, such as faecal extracts and bile. Other benefits of the system described, include increased throughput, in-part due to the fast high efficiency separations of UPLC, but also due to the reduced drying times associated with small volume fractions. In addition, the enhanced chromatographic efficiency of UPLC has the potential to increase the resolution between radioactive metabolites of interest and endogenous interference, thereby reducing matrix effects. Such interference from significant amounts of protein, salt, or other endogenous material, often associated with biological samples, can generate chemical quenching, or alternatively can act as a physical barrier between the radioactive substance and the scintillant.

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