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High volume injections of biological samples for sensitive metabolite profiling and quantitation

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ABSTRACT

An online preconcentration approach was developed allowing the injection of very high volumes of biological samples, thereby greatly increasing sensitivity while maintaining LC resolution. The approach was applied to the analysis of radioactive samples from both *in vitro* and *in vivo* metabolism studies where typically the concentration of radioactivity given is often limited, while sample volume is usually not. The described online preconcentration approach reduces sample preparation and, therefore, also the risk for degradation and recovery issues often seen with offline preconcentration methods. In addition to facilitating the identification and profiling of low level metabolites within a sample, the described approach also provides robust quantitative analysis of samples derived from a range of biological matrices. The application of this approach is illustrated on real life samples from different matrices and containing drugs and metabolites with a wide variety in polarity, more specifically the analysis of extracts derived from an *in vitro* hepatocyte incubation, 36 mL of blood/acetonitrile (1/1, v/v; 28 dpm/mL) and 72 mL of urine/methanol (9/1, v/v; 208 dpm/mL).

1. Introduction

Sensitivity is often one of the most critical parameters in analytical chemistry. Increased sensitivity enables more to be done with the same or less sample, allows new applications or results with better signal-to-noise and, consequently, better accuracy. Usually this is achieved by advancements in detector technology which is in general easily implemented and does not affect our daily work flows. If only a small fraction of the available sample is used, sensitivity can alternatively be gained by increasing the volume of sample being analyzed. The analysis of pollutants in water is a typical example where this approach is applied [1–3].

Also metabolite profiling of radiolabeled substrates would greatly benefit from analysis of larger volumes of sample. Radiotracer technology (^{14}C or ^{3}H) is still the method of choice to study the *in vivo* disposition of a new drug as it allows making up a mass balance and enables the quantitative detection of the parent drug and all of its metabolites in complex matrices without the need for authentic standards. The concentration of radioactivity that can be given especially in human *in vivo* studies is limited, while sample volume (urine, faeces, plasma) is usually not. Therefore, larger volumes (100–500 μL) are more routinely injected upon radio-UHPLC analysis than commonly applied for UHPLC analyses [4]. Since this is only a fraction of the available sample, a sample preconcentration step *via* protein precipitation, centrifugation and drying and reconstitution of the supernatant in an appropriate solvent is common practice in radioactive metabolite profiling, especially for plasma sample analysis. This offline sample preparation is not only time consuming, but also adds a risk for degradation and often results in low recoveries usually the result of partial dissolution in the reconstitution solvent used because of its very limited volume in relation with the original volume of supernatant and container.

Here an online sample concentration approach is described that overcomes these issues. The online solid phase extraction (SPE) or turboflow methods most commonly used for online preconcentration, aim at selectively retaining one or several compounds from the same class [5–8]. A preconcentration step applied for metabolite profiling should, in contrast, be relatively unselective and generic since every metabolism study sample can be different and potentially contain new and unknown analytes. It should also be able to retain compounds with a wide range of polarities since metabolites tend to be more polar, and often substantially more polar (*e.g.*, phase II metabolites) than the parent drug. The downside of this broadly applicable approach is that sample clean-up during online concentration is limited. This is not a real issue with radioactive detection since its specificity is extremely high and matrix effects are limited or absent.

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The methodology described here was optimized and used for radiolabeled samples and so any sample losses due to column breakthrough or adsorption/carry over could be easily followed. If loss of radioactivity was experienced, small method adjustments were evaluated and implemented to overcome these losses. This methodology is routinely being applied in our laboratory for the analyses of samples (mainly radioactive) where the injection of very large volumes allows any recovery or stability issues observed (or expected) with an offline preconcentration approach to be overcome, the saving of time by limiting sample preparation, extending the life time of the analytical column, and the analysis of samples with online RAD detection instead of offline collection and counting. We will share our experiences *via* some real life examples of analyses of *in vitro* and *in vivo* samples.

2. Experimental

2.1. Chemicals and materials

Methanol and acetonitrile (both HPLC grade) were supplied by Merck (Darmstadt, Germany). Ammonium acetate was purchased from VWR International (Leuven, Belgium). Acetic acid and ammonia (25%), used to bring the mobile phases to the desired pH, were obtained from Merck (Darmstadt, Germany). For preparation of the samples, dimethylsulfoxide (DMSO) from Merck (Darmstadt, Germany) was added. High purity water was used throughout and was obtained by purifying doubly distilled water in a Milli-Q system (Millipore, Bedford, MA, USA). Compound 1, Compound 2 and ibrutinib are proprietary Janssen development compounds synthesized and labelled with ^{14}C in-house. The chemical structure of Compound 1 and ibrutinib is shown in Fig. 1. Compound 2 is a weak base, MW ± 400, small molecule drug compound for which the structure cannot be disclosed.

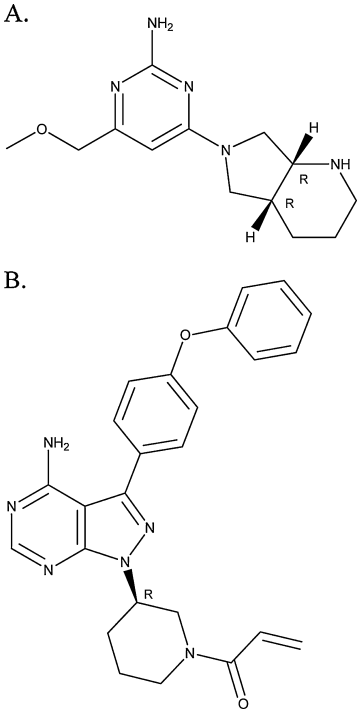

Fig. 1. Chemical structures of (A) compound 1 and (B) ibrutinib.

2.2. Sample preparation

2.2.1. Preparation of standards and calibration curves

A 7 mL stock solution containing 1.05·E7 dpm (disintegrations per minute) of ^{14}C labelled Compound 1 and 2 was prepared in DMSO. This stock solution was further diluted with DMSO to give a series of five calibration solutions ranging from 2000 to 75,000 dpm/injection volume (150 μL for compound 1 and 100 μL for compound 2). The corresponding radioactive levels were calculated based on liquid scintillation counting (LSC) (Tricarb 2100TR, Perkin Elmer, Massachusetts, USA) in triplicate. The five calibration solutions were injected in duplicate to create a calibration curve for quantification of the analytical injections. The set of calibration standards used for the preparation of the high volume injected calibration curves, was made by diluting the analytical solution further with DMSO until the desired concentration.

2.2.2. Human hepatocyte incubations

Compound 1, ^{14}C-labelled, was incubated at 5 μM (6.3·E5 dpm/mL) for 48 h in cryopreserved plated hepatocytes. The cell incubations (500 μL) were extracted with DMSO (500 μL), vortexed, sonicated and centrifuged (10 min at 16,060 × *g*) to remove particulate matter. A 400 μL aliquot of the resulting supernatant was injected for analysis. The total radioactivity injected was determined to be 136,500 dpm using LSC.

2.2.3. Preparation of human blood

Blood samples derived from a human ^{14}C mass balance study of ibrutinib dosed at 8.88·E7 dpm (140 mg dose) were prepared for analysis. The samples were pooled (6 subjects, 4 mL per subject) per time point across the subjects, proteins were precipitated by the addition of one volume of acetonitrile (24 mL), vortex mixed and centrifuged at 2125 × *g* for 20 min at 4 °C. The resulting supernatant was then loaded, in 9 injection cycles of 4 mL, directly onto the online preconcentration-UHPLC system. The injected amount of radioactivity was approximately 1000 dpm, determined by spiking the sample to a Lumaplate (Perkin Elmer, Massachusetts, USA) and counted by a microplate scintillation counter (MSC) (Topcount NXT, Perkin Elmer, Massachusetts, USA).

2.2.4. Preparation of human urine

Human urine was obtained from a human ^{14}C mass balance study of Compound 2 dosed at 2.95·E6 dpm (30 mg dose). Urine was centrifuged (20 min at 2125 × *g*) and 10% methanol (9:1, v/v, 9 mL) was added to an aliquot of the supernatant (81 mL). The resulting sample was loaded onto the online preconcentration system (18 × 4 mL injection). The total injected radioactivity present in the urine supernatant was determined to be 13,477 dpm using a Tricarb 2100TR LSC.

2.3. Instrumentation

In the following section, the instrumentation is described based on its order of use in the analysis of the human hepatocyte sample. The parameters applied in the other examples are summarized in Table 1.

An Open Architecture Acquity UPLC system, equipped with a CTC PAL auto sampler (Waters, Manchester, UK) containing 3 high pressure 6-port switching valves, was used to place the sample in the 500 μL preparative loop (Fig. 2). Two Agilent 1100 HPLC pumps (Santa Clara, USA) loaded the sample on the trapping column (Waters X-Bridge BEH C-18; 50 mm × 4.6 mm ID; 3.5 μm) kept at room temperature. The first pump was used for sample loading at a flow rate of 0.5 mL/min with water/methanol (9/1; v/v) and the second pump, equipped with 25 mM ammonium acetate pH 10, was used for in flow dilution at 5 mL/min. After preconcentration,