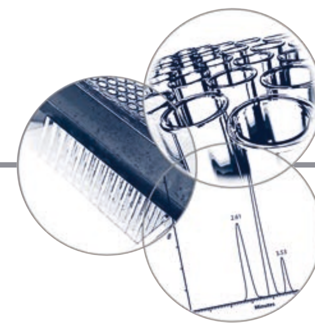

Multiplexed extraction and quantitative analysis of pharmaceuticals from DBS samples using digital microfluidics

Background: Dried blood spot (DBS) sampling is emerging as a valuable technique in a variety of fields, including clinical and preclinical testing of pharmaceuticals. Despite this popularity, current DBS sampling and analysis processes remain laborious and time consuming. Digital microfluidics, a microscale liquid-handling technique, characterized by the manipulation of discrete droplets on open electrode arrays, offers a potential solution to these problems. **Results:** We report a new digital microfluidic method for multiplexed extraction and analysis of pharmaceuticals in DBS samples. In the new method, four DBS samples are extracted in microliter-sized droplets containing internal standard, and the extract is delivered to dedicated nanoelectrospray ionization emitters for direct analysis by tandem mass spectometry and selected reaction monitoring. **Conclusion:** The new method allows for an order of magnitude reduction in processing time and approximately three-times reduction in extraction solvent relative to conventional techniques, while maintaining acceptable analytical performance for most drugs tested.

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Dried blood spot (DBS) sampling, in which a pin prick of blood is collected and dried on a paper substrate prior to analysis, is becoming popular for a wide range of applications [1–7]. One field that is emerging as a beneficiary of DBS sampling is preclinical and clinical testing of pharmaceutical candidates, which includes animal toxicokinetic (TK) [8], pharmacokinetic PK, drug metabolism [9–11] and therapeutic drug monitoring studies [12–14]. In these applications, DBS sampling enables a reduction in sample processing, transportation and storage costs [15], and also has ethical benefits, which helps patient recruitment in clinical studies [13,16]. DBS sampling is gaining particular favor for preclinical TK studies of new chemical entities, as it is consistent with the '3Rs' of animal experimentation (replacement, reduction and refinement) [17]. With DBS samples, it is possible to collect 10–20 µl blood samples at each time point, which is much less invasive than the standard techniques that can require more than 0.5 ml per time point (which moreover may require additional manipulation of the animal, e.g., 10 min warming period prior to sampling) [16,17]. DBS sampling allows for a full concentration, time profile on each of the main study animals, which eliminates the need for satellite groups [17], increasing data quality and alleviating ethical concerns [8]. The benefits of DBS sampling are even more important in the development of pediatric pharmaceuticals, as the young subjects have reduced amounts of blood available for analysis (and may have different PK characteristics than those of adults) [18,19].

Unfortunately, DBS analysis remains a laborious, time-consuming task, requiring a disk to be punched, the analytes to be extracted from the punch, the analytes to be mixed with internal standard (IS), and (in some cases) derivatized prior to analysis by HPLC and **selected reaction monitoring (SRM)** by tandem mass spectometry (MS/MS) [15]. This processing regimen represents a significant barrier to widespread adoption of DBS sampling and analysis. A number of strategies have been developed to address these throughput issues [20], including direct desorption techniques such as desorption electrospray ionization MS [21–23], electrospray directly from DBS sample paper [24], and the direct elution of analytes from DBS samples using a variety of techniques such as the CAMAG thin-layer chromatography MS interface [15,25,26]. Despite the growing interest in DBS samples, there are few automated solutions available [101–104], particularly for 'direct analysis' techniques that eliminate the HPLC separation associated with conventional analysis.

We recently described the first microfluidic techniques useful for extracting and quantifying analytes in DBS samples [27,28]. These techniques are powered by **digital microfluidics** (DMF), a fluid handling technique in which discrete droplets (~28 µl) of samples and reagents are

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Key Terms

Dried blood spot (DBS): Microscale blood sampling technique that is rapidly gaining popularity, whereby small volumes of blood are spotted onto cellulose matrix cards and stored at room temperature.

Selected reaction monitoring (SRM): The selection, fragmentation and detection of particular precursor/product ions for the purposes of quantification.

Digital microfluidics: Lab-on-a-chip technology enabling the manipulation of small droplets of liquid on a 2D-array of electrodes.

manipulated (i.e., dispensed from reservoirs, split, merged and mixed) on an open surface by applying a series of electrical potentials to an array of electrodes [29,30]. Droplet actuation in such systems is driven by electromechanical forces [31,32] generated on free charges in the droplet meniscus (in case of conductive liquids), or on dipoles inside of the droplet (in case of dielectric liquids). Moreover, DMF can be used to actuate a wide range of organic solvents [33] (e.g., methanol, acetonitrile, acetone and chloroform), making it suitable for extraction from solid matrices. DMF has recently emerged as a powerful method for sample processing for analysis by MS [34]. In initial work, DMF was applied to analyzing biomarkers for amino acid metabolism disorders in newborns [27,28]. These methods represent an important step forward, but they are limited to serial analysis of one DBS sample at a time. Here, we describe the development of a new automated DMF direct analysis method for parallel processing and quantification of pharmaceuticals for clinical testing, and compare its performance (assessed in a university setting in Toronto, Canada) to that of conventional laboratory processing (assessed in a pharmaceutical analysis laboratory in Ware, UK). We propose that the method presented here may represent a step toward a new tool for preclinical and clinical testing in the pharmaceutical industry.

Experimental

Reagents & materials

Unless otherwise specified, reagents were purchased from Sigma Chemical (ON, USA). Compounds used as analytes and IS were sourced as follows: ibuprofen, $^{2}H_{3}$ ibuprofen, acetaminophen and proguanil were obtained from Sigma-Aldrich (Pool, UK), $^{2}H_{4}$ acetaminophen, $^{2}H_{4}$ proguanil, simvastatin and $^{2}H_{3}{}^{13}C$ simvastatin were obtained from Toronto Research Chemicals (NY, Canada), benzethonium chloride was obtainedfrom Fisher Scientific (Loughborough, UK), and sitamaquine, $^{2}H_{10}$ sitamaquine and SB-243213 were supplied by GSK (Stevenage, UK). Parylene-C dimer was from Specialty Coating Systems (IN, USA), and Teflon-AF was purchased from DuPont (DE, USA). All working solutions were prepared using HPLC grade methanol.

DBS samples for analysis by DMF and conventional approaches were prepared in Ware, UK using methods described previously [13,35]. Briefly, stock solutions of six drugs (Table 1) were prepared in dimethylformamide at 1 mg/ml for all compounds other than ibuprofen, which was prepared at 10 mg/ml. Working standards at suitable concentrations were made-up in acetonitrile/water (1:1, v/v), which were diluted into control rat blood from B&K Universal (Hull, UK) with a maximum of 5% non-matrix solvent. Calibration standards (in blood) were prepared at the following concentrations: 5, 20, 100, 800 and 1000 ng/ml (sitamaquine and proguanil); 100, 400, 2000, 16,000 and 20,000 ng/ml (benzethonium chloride and acetaminophen); 25, 100, 500, 4000 and 5000 ng/ml (simvastatin): and, 5000, 20,000, 50,000, 400,000 and 500,000 ng/ml (ibuprofen). For each analyte, a single concentration blind QC sample (in blood) was also prepared. 15 µl samples of blood were pipetted onto Ahlstrom 226 (untreated) DBS cards and allowed to dry at room temperature for at least 2 h. Some samples were evaluated in-house by HPLC–MS/MS and others were mailed to Toronto for direct analysis by DMF. The IS corresponding to each drug was a stable deuterated form of the drug, except for benzethonium chloride, for which SB-243213 was used. Individual stock IS solutions were prepared in dimethylformamide (10 mg/ml for ibuprofen, 1 mg/ml for all other compounds). For experiments in Ware, a single working IS solution was prepared in methanol at 20 ng/ml for sitamaquine, 10 ng/ml for proguanil, 10 ng/ml for benzethonium chloride, 1 µg/ml for ibuprofen, 1000 ng/ml for simvastatin and 50 ng/ml for acetaminophen. For experiments in Toronto, six different working IS solutions were prepared in methanol at 4 ng/ml for sitamaquine and proguanil, 100 ng/ml for benzethonium chloride and 4 µg/ml for ibuprofen. These working solutions were used for extraction and analysis, as described below.

DMF device fabrication

DMF devices were fabricated in the University of Toronto Nanofabrication Centre (ON, Canada), using a transparent photomask printed at Pacific Arts and Design (ON, Canada). DMF device bottom plates were formed from chromium coated glass substrates purchased from Telic Company (CA, USA), and top plates were formed from indium tin oxide (ITO) coated glass substrates purchased from Delta Technologies Ltd (MN, USA). Bottom plate electrodes were formed and coated with Parylene-C, and bottom and top plates both were coated with Teflon-AF, as described previously [36].

Table 1. Test panel of drugs.

Drug	MW (Da)	pKa	LogP	Structure
Sitamaquine (base) – antimalarial	343	2.7 (NH) 10.3 (NR3)	5.59	
Proguanil (base) – prophylactic antimalarial	253	9.6 (NH) 10.3 (NH)	2.53	
Benzethonium chloride (quaternary amine) – topical antimicrobial	412	–	4.29	
Ibuprofen (acid) anti-inflammatory/analgesic	206	4.9 (OH)	3.68	
Acetaminophen (neutral) – analgesic	151	9.4 (OH)	0.49	
Simvastatin (neutral) – hypolipidemic drug	418	–	4.48	

The device design (Figure 1) features four extraction zones comprising three thin actuation electrodes (5 × 2 mm) connected to four large actuation electrodes (7 × 7 mm), with interelectrode gaps of 40 µm. Devices were assembled with an unpatterned ITO–glass top plate and a patterned bottom plate, such that the 102 × 32 mm top plate was aligned with the outer-edges of the reservoir electrodes on the bottom plate. The two plates were separated by a spacer formed from five pieces of double-sided tape (total spacer thickness ~450 µm). With these dimensions, droplets covering the 7 × 7 mm electrodes were approximately 28 µl.

Driving potentials of approximately 150 V_{RMS} were generated by amplifying the sine wave output of a function generator (Agilent Technologies, CA, USA) operating at 15 kHz. The application of driving potentials to the device was managed using an automated feedback control system described previously [28,37]. Reagents were loaded onto a DMF device by pipetting an aliquot (~28 µl) onto the bottom plate at the edge of the top plate, and simultaneously applying driving potential to the appropriate reservoir electrode (relative to the ITO electrode on the top plate) to draw the fluid into the reservoir [38,39]. Thereafter, droplets were manipulated by applying the driving potential to sequential actuation electrodes on the bottom plate relative to the ITO electrode on the top plate.

DMF-nanoESI-MS interface

DMF devices were interfaced to MS using pulled glass nanoelectrospray ionization (nESI) emitters sandwiched between the top and bottom plates using methods similar to those described previously [28]. Briefly, four nanoESI emitters (~5 cm long, 360 µm outer diameter., 50 µm inner diameter, 30 µm tip inner diameter) (New

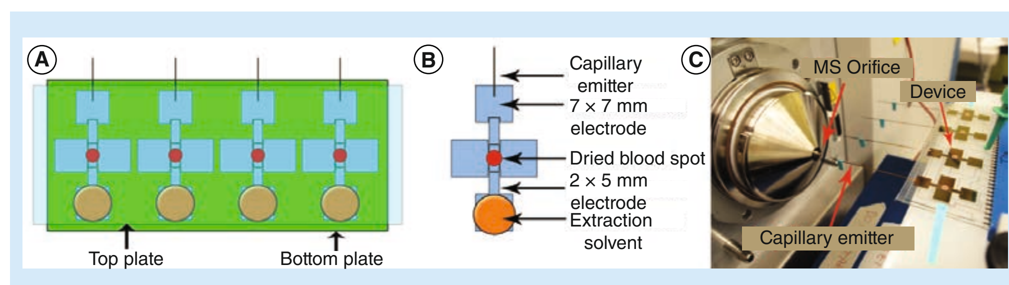

Figure 1. Digital microfluidic device used for extraction of drugs from DBS punches. **(A)** Device, which features four independent digital microfluidics modules mated to pulled-glass capillary nanoelectrospray ionization emitters for MS. **(B)** Schematic of a single module, which features four 7 × 7 mm and three 5 × 2 mm actuation electrodes. As shown, the DBS punch is positioned on top of the central 5 × 2 mm electrode. **(C)** Picture of a digital microfluidics device interfaced with a mass spectrometer.

Objective Inc., MA, USA) were inserted between the two plates of the DMF device. The device was then positioned in front of the MS such that the tip of one of the capillaries was approximately 3 mm away from the orifice of the MS inlet. After a droplet was driven to the entrance of a pulled-glass emitter, it filled by capillary action in <1 second. (Over the course of hundreds of experiments, emitters were observed to fill 100% of the time, with no noticeable variation in filling time.) A high DC potential (±1.7–2.2 kV in positive or negative mode) was applied to the ITO-coated top plate of the DMF device to generate a nanoelectrospray into an LTQ Mass Spectrometer (Thermo Scientific). To switch between emitters, devices were manually translated horizontally in front of the mass spectrometer.

DMF-driven DBS processing & analysis

Punches (3 mm diameter) from DBS cards were generated using a Harris Uni-Core biopsy punch tool purchased from Sigma–Aldrich (Oakville, ON) and were analyzed by DMF-MS/MS in Toronto. In typical experiments, four punches were positioned on top of the four central electrodes on the bottom plate of the device. The top plate was then positioned on the device and 28 µl aliquots of methanol containing the appropriate IS were loaded into each of the four reservoirs. The droplets were then actuated onto the DBS punches, and cycled ten-times back-and-forth between the large electrodes adjacent to the punches. The droplets were then incubated on the DBS punches for 5, 10, 15 or 30 min (repeatable within ~10 s) at room temperature, and then actuated onto the electrodes adjacent to the capillary emitters for analysis.

Analytes were ionized in positive mode for benzethonium chloride, sitamaquine, proguanil, acetaminophen and simvastatin, and negative mode for ibuprofen. High purity (99.995%) helium gas (135 ± 70 kPa) was used for collision activated dissociation, and mass transitions of 412 to 320 and 429 to 228; 344 to 271 and 354 to 271; 254 to 170 and 258 to 174; 152 to 110 and 156 to 114; 419 to 285 and 423 to 285; and 205 to 161 and 208 to 164, were monitored for benzethonium chloride, sitamaquine, proguanil, acetaminophen, simvastatin and ibuprofen, and their corresponding IS, respectively. Each drug was analyzed separately, and for each analysis, two *m/z* transitions were monitored in series: that of the analyte of interest and that of the corresponding IS. The ratios of peak intensities (drug: IS) of the product ions were recorded and used for quantification. Spectra were collected as an average of ten acquisitions using Thermo Finnigan™ Xcalibur® software (Version 2.0), and at least four samples were evaluated for every condition recorded. To form calibration curves, concentration-dependent data were fit with linear regressions using IGOR Pro (Version 5.0.4.8, WaveMetrics, Inc., OR, USA).

Conventional DBS processing & analysis

DBS samples were processed and analyzed in Ware by HPLC–MS/MS using qualified methods, described in detail previously [35]. Prior to their use for the analysis of test samples here, the methods were requalified in three consecutive LC–MS/MS analytical runs to verify their linearity, precision and accuracy. Briefly, 3 mm diameter disks were punched from the center of the DBS cards into clean tubes, followed by a 1 h extraction with 100 µl IS working solution. The samples were then vortex mixed for approximately 20 s, and centrifuged for 1 min at 3000 × g. The supernatant was then transferred

to a clean tube for analysis by HPLC–MS/MS. The HPLC system comprised a CTC HTS PAL autosampler (Presearch, Hitchin, UK) with fast wash and an Agilent 1100 binary pump (CA, USA) with integrated column oven and divert valve. The MS was a triple quadrupole API-5000 (Applied Biosystems/MDS Sciex, ON, Canada) equipped with a Turbo Ionspray ion source. The collision gas (collision activated dissociation), nebuliser gas (GS1), and auxiliary/turbo gas (GS2) were all ultra-high purity Nitrogen. MS source conditions were optimized to give the maximum response for a given analyte/assay. Concentrations of test compounds were determined from the peak area ratios of analyte to IS using Analyst software (Version 1.4.2, Applied Biosystems/MDS Sciex, ON, Canada).

Methods similar to those reported previously were used to analyze all analytes except acetaminophen [15]. Briefly, the method included a Hypurity C18 3 µm HPLC column (Thermo Fisher, Loughborough, UK, 50 mm long 2.1 mm internal diameter), a flow rate of 1000 µl/min, column temperature of 60 °C, run time of 3.8 min, and gradient chromatography employing mobile phases methyl ammonium acetate (10 mM, pH 4.2) (A) and acetonitrile (B). Following sample injection, the mobile phase was held at 95% A for 0.1 min. A ballistic gradient to 20% A at 1.0 min was followed by an isocratic period at 20% A to 3 min. The mobile phase was then returned to 95% A by 3.2 min and was held until 3.8 min, prior to injection of the next sample.

A method similar to that reported previously was used to analyze acetaminophen [15]. Briefly, a YMC AQ C18 3 µm HPLC column (Dinslaken, Germany, 50 mm long 4 mm internal diameter), a flow rate of 800 µl/min, column temperature of 60 µC, run time of 2.5 min, and gradient chromatography employing the mobile phases ammonium acetate (1 mM, native pH) (A) and acetonitrile (B). Following sample injection, the mobile phase was held at 95% A for 0.08 min. A ballistic gradient to 0% A at 1.08 min was followed by an isocratic period at 0% A to 1.25 min. The mobile phase was then returned to 95% A by 1.26 min and was held as this composition until 2.5 min, prior to injection of the next sample. The same SRM transitions were monitored for all drug/IS pairs as outlined in the DMF-driven DBS analysis section, above.

For each test compound, six replicates of the blind QC were run, bracketed by two (five point) calibration lines plus total blank (matrix only) and blank (blank control matrix plus IS) samples. Calibration plots of analyte/IS peak area ratio versus the nominal concentration of the analyte in blood were constructed (using Applied Biosystems/MDS Sciex Analyst software v1.4.2), and a weighted $1/x^2$ linear regression was applied to the data for all analytes. Internationally recognized acceptance criteria was applied to all analytical batches to ensure their validity [40]. Post-analysis, the nominal blind QC concentrations were unblinded and a mean accuracy (% bias) and precision (%CV) value was calculated from the six replicate blind QC for each analyte.

Results & discussion

■ Device & test candidates

The primary goal of this work was to develop a new DMF method for multiplexed, quantitative analysis of drugs in DBS samples. As shown in Figure 1, a new device design was generated bearing four modules to allow for the extraction of four samples in parallel. Each module comprises four 7 x 7 mm electrodes and three 2 × 5 mm electrodes, and each module is interfaced to a dedicated nESI emitter. To our knowledge, this is the first DMF device that is integrated with multiple ionization sources. In a typical experiment, DBS samples are manually punched from the card and positioned on the top of the central 2 × 5 mm electrodes using tweezers, the top plate is placed onto the device (separated by spacers of double-sided tape), and the device is connected to an automated droplet **digital microfluidic control system**, which is described in detail elsewhere [28,37]. During the extraction process, the DBS sample maintains contact with the top and bottom plates such that it remains stationary. The control system makes regular impedance measurements and uses this information to maintain high-fidelity control over droplet position, which is particularly important for manipulating droplets onto and off of the absorbent DBS punches.

A typical experiment is depicted in Figure 2. An aliquot of solvent (~28 µl) is loaded onto the device (Figure 2A), driven onto the DBS punch, shuttled back and forth (Figure 2B & C) and incubated (see the following section for discussion of incubation time optimization). The droplet is then driven to the nESI emitter (Figure 2D), where, after spontaneously filling the emitter by capillary action, an electrospray is initiated by applying a potential to the top-plate electrode. In this scheme, the four extractions

Key Term

Digital microfluidic control system: Electronic interface that provides automated control over droplet position on digital microfluidics devices; an 'open-source' digital microfluidics control system is available online at http://microfluidics.utoronto.ca/dropbot.

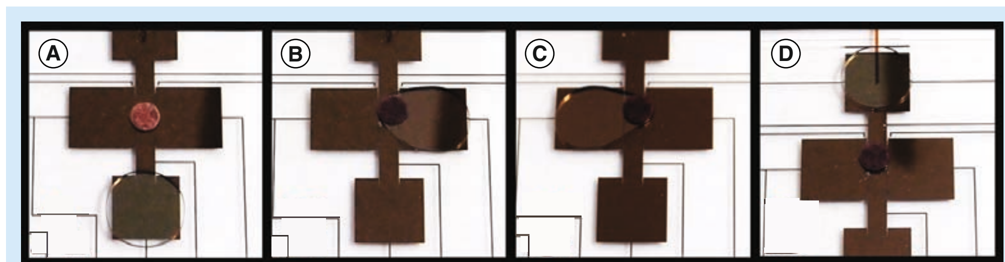

Figure 2. Digital microfluidic DBS extraction. (A) A reservoir is filled with extraction solvent (methanol-containing IS). **(B & C)** The solvent is then driven onto the DBS and actuated back-and-forth before incubating. **(D)** After incubation, the droplet is moved to the final electrode, where it fills the pulled glass emitter by capillary action.

can be performed in parallel, but the nESI-MS analyses for four DBS punches are conducted serially by translating the device to position each emitter in front of the MS inlet. If desirable, in the future, methods for multiplexed ionization and detection [41] might be adapted to allow for parallel analysis.

A panel of six drugs was selected to test the new DMF extraction and analysis system (Table 1). The panel includes two secondary amines used for their antimalarial properties (sitamaquine and proguanil), a quaternary amine used as a topical antimicrobial agent (benzethonium chloride), a carboxylic acid used as an analgesic (ibuprofen), and two neutral compounds, a mild analgesic (acetaminophen) and a hypolipidemic agent (simvastatin). These drugs were spiked into rat blood at physiologically relevant concentrations and were used to form DBS samples for analysis using the method shown in Figure 2. This panel was chosen in part because it has been used previously in a range of direct analysis techniques, including direct elution [15], desorption electrospray ionisation [23], and paper spray [24]. Previous studies have shown that direct analysis techniques that do not use LC have been unable to match the sensitivity of conventional LC–MS/MS analysis for at least some of the compounds in this suite, with the neutral compounds (acetaminophen and simvastatin) being the most problematic [20].

■ MS/MS analysis & optimization

The traditional method used for offline extraction and HPLC–MS/MS analysis of pharmaceuticals in DBS samples requires several hours per sample [15], although the extraction phase can be done for multiple samples in parallel. Typically, several hundred DBS samples may be extracted in parallel, an activity that requires several hours to complete, followed by approximately 5 h of HPLC–MS/MS analysis time per 100 samples. In developing the method described here, we strove to improve upon this by (a) eliminating the HPLC separation (i.e., direct analysis), and (b) taking advantage of the inherent benefits of microfluidics including reduced reagent use and method integration. For (a), quantitation without HPLC required careful attention to IS and SRM transitions, which are described in the methods section. A further consideration is that metabolites of some structures (*N*-oxides, acyl glucuronides and sulfates) may undergo transformation back to the parent drug in the source region of the MS. Methods were developed for the analysis of each drug in the panel using the mass transitions described above. The DMF/direct analysis technique worked well for four drugs (sitamaquine, proguanil, benzethonium chloride and ibuprofen), but failed to yield reproducible signals for two (acetaminophen and simvastatin), which suggests that the new DMF-MS/MS protocol may face the same challenges as other direct MS/MS techniques for neutral compounds [20]. Representative spectra for sitamaquine are shown in Figure 3A, including strong SRM peaks for both the native drug (in the DBS) at m/z 344 → 271 and the deuterated IS (in the extraction solvent) at m/z 354 → 271. Sitamaquine, proguanil, benzethonium chloride and ibuprofen were used in the remaining experiments.

For the second improvement (b) associated with the DMF method (shortened extractions), four different incubation times were evaluated: 5, 10, 15 and 30 min. In separate experiments for each drug, four replicate DBS punches were extracted and analyzed per extraction time. The concentrations selected for these experiments were pharmaceutically relevant values: 100 ng/ml for sitamaquine and proguanil, 50,000 ng/ml for ibuprofen, and 2000 ng/ml for benzethonium

chloride. Figure 3B summarizes the results of the extraction time optimization experiment. As shown, signal strength generally increases as the extraction time is increased, which suggests that longer extraction times are better for sensitive analyses. But this benefit is offset by an increase in variation associated with the longer extraction times, most notably with benzethonium chloride and ibuprofen. We hypothesize that the increased variation at longer incubation times is related to evaporation. The method reported here makes use of devices that are open to the environment; by the end of a 15 or 30 min extraction time, evaporation becomes significant, particularly for solvents (such as methanol, used here) that have a low boiling point. The inclusion of IS in the extraction solvent mitigates this problem to a degree, but after long incubation times, it was occasionally observed that the droplet had evaporated to an extent that droplet actuation was unpredictable (resulting in longer than anticipated extraction times).

In the future, if increased sensitivity (related to long extraction times) is desired, a number of different strategies might be used to reduce evaporation, including a temperature-controlled chamber [42] or the use of a gasket to seal the device [43]. A third strategy might include operating the device with filler media other than air (e.g., silicone oil [44]), but exposure to oil is not a perfect match for applications (such as the one reported here) requiring analysis by MS. Regardless, for the work reported here, the variation was sufficiently low for incubation times of 5 min (%CVs: sitamaquine – 17.1%, proguanil – 10.4%, benzethonium chloride – 10.8%, ibuprofen – 35.6%) and 10 min (%CVs: sitamaquine – 11.8%, proguanil – 16.8%, benzethonium chloride – 7.8%, ibuprofen – 31.0%) for quantitative analysis, and 5 min incubation was used for the remainder of the work described here.

The total time required to extract and analyze a single DBS sample (including MS/MS analysis time) is approximately 6.5 min, representing a tenfold improvement relative to the standard method used in industry. This number does not take multiplexing into account; for example, in conventional analysis, DBS samples can be extracted in parallel in well plates, which reduces the amount of time required per data point. The DMF system described here is also multiplexed, allowing for four parallel extractions, and we propose that higher levels of multiplexing should be achievable in the future, given the recent report of DMF devices with 4096 individually addressable electrodes [45]. In addition, the solvent volume used in the DMF method (28 µl per DBS) is substantially lower than that used in the standard method (100 µl extraction solvent per DBS plus 1.5 ml LC–MS/MS solvent). The DMF extraction volume was chosen arbitrarily for the work reported here; we propose that in future experiments with greater control of evaporation (as described above), even greater reductions in volume might be achieved. For these future experiments, we propose that the minimum volume required is two-times the volume that can absorb into a DBS punch (e.g., for a 3 mm diameter punch, the minimum volume is ~6 µl).

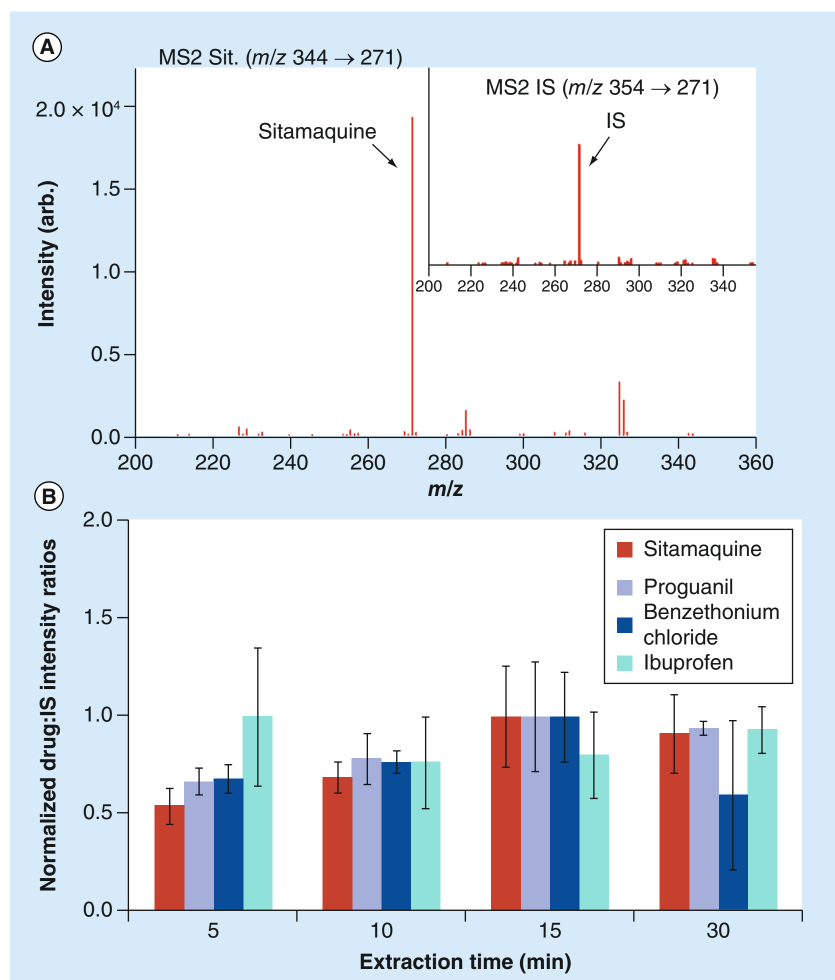

Figure 3. Digital microfluidic extraction and analysis of pharmaceuticals from DBS samples. (A) Representative positive ion tandem mass spectra of sitamaquine collected via nanoelectrospray (*m/z* 344 → 271, in the DBS punch) (main panel) and deuterated IS (IS, *m/z* 354 → 271, in methanol) (inset). **(B)** Peak intensity ratio of drug:IS as a function of on-chip incubation time.
Red: sitamaquine (100 ng/ml); blue: proguanil (100 ng/ml); purple: benzethonium chloride (2000 ng/ml); green: ibuprofen (50,000 ng/ml).
The error bars represent ±1 SD, n = 4.
Please see colour figure at www.future-science.com/doi/full/10.4155/BIO.13.311

Quantitative analysis

The DMF/direct analysis method described above was applied to quantify sitamaquine, proguanil, benzethonium chloride and ibuprofen. Calibration curves were generated by plotting the intensity ratios of drug product ions relative to those of IS as a function of drug concentration in a series of DBS samples (FIGURE 4). As shown, the curves generated by DMF/direct analysis are linear over multiple orders of magnitude, with R^2 values of 0.9999, 0.9974, 0.9964 and 0.9977, respectively. For comparison, calibration plots of analyte/IS peak area ratio versus the nominal concentration of the analyte in blood were also constructed using conventional HPLC–MS/MS methods. Curves were linear over the calibration ranges, with R^2 values of 0.9954, 0.9969, 0.9885 and 0.9962 for sitamaquine, proguanil, benzethonium chloride and ibuprofen, respectively.

Replicate blind QC DBS samples were analyzed using the DMF protocol in Toronto and the traditional macroscale protocol at GSK R&D in Ware. TABLE 2 summarizes the results. For sitamaquine and proguanil, the DMF and conventional protocols performed similarly, with measured accuracies relative to the unblinded concentrations of 90.0% and 101% (DMF) and 109% and 104% (conventional). This is striking, given that the analyses were performed using such different methodologies and were carried out by different operators working in different continents. In contrast, the conventional protocol was more accurate than the DMF protocol for the quantification of benzethonium chloride and ibuprofen, with measured accuracies of 112% and 98.8% (conventional), and 66.9% and 119.5% (DMF), respectively. It is possible that the benzethonium chloride and ibuprofen in the DBS samples that were analyzed by DMF (that were mailed from

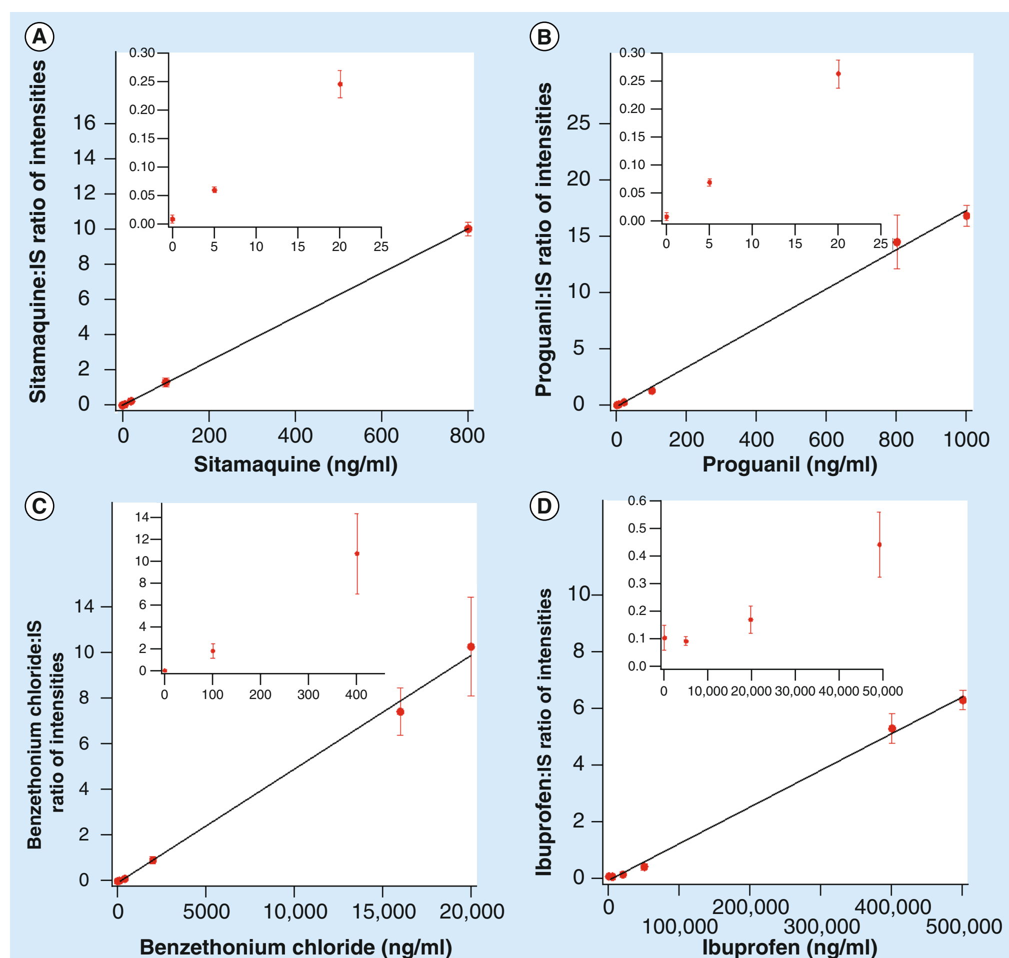

Figure 4. Calibration curves for drugs extracted from DBS samples in digital microfluidics devices. (A) Sitamaquine, R^2: 0.99997. **(B)** Proguanil, R^2: 0.99741. **(C)** Benzethonium chloride, R^2: 0.9964. **(D)** Ibuprofen, R^2: 0.9977. Insets highlight the low-concentration data. The error bars represent ±1 SD, n = 4.

the UK to Toronto) were subject to temperature and humidity changes, which are known to degrade some analytes [46]. But it is also likely that simple changes in methodology could improve these results, for example, tuning the extraction solvent to favor the solubility of weak acids [47] or quaternary amines [48], or adjusting the solvent composition after extraction for higher ionization efficiency. Similarly, these steps might enable quantification of acetaminophen and simvastatin.

To summarize, the data presented here demonstrate that the DMF-DBS direct analysis technique is: well suited for quantifying sitamaquine and proguanil; compatible with quantifying benzethonium chloride and ibuprofen (but would benefit from further optimization; and not suitable for quantifying acetaminophen and simvastatin without a significant change in methodology.

The device and method described here represents a baseline for using DMF as a multiplexed sample processing tool for clinical or preclinical evaluation of pharmaceutical agents in DBS samples. In the future, we propose that there are a number of potential device/instrument-level improvements that could be made to improve the technique (bringing it closer to being universal) including integration with DMF-driven SPE modules for purification and concentration [49], and/or interfacing DMF processing with HPLC or other separation techniques [50,51]. The current popularity of DBS sampling is driving significant innovations, including the recent development of robotic platforms capable of DBS sample analysis [105]. These robotic systems (while still requiring some fine-tuning) will undoubtedly be useful in large, well-funded laboratories. But the work presented here suggests that DMF may present an attractive alternative for laboratories that do not have access to robotic systems, with the added advantages of reduced extraction time and solvent/reagent usage. The simplicity of the approach presented may also be able to be adapted to the placement of analytical tools closer to the patient (e.g., in the hospital) rather than in centralized analytical facilities. This may be of particular importance where rapid decision making is required.

Future perspective

DMF is emerging as a versatile sample processing platform to combine with MS [34]. We report a new DMF method for multiplexed extraction of pharmaceuticals from DBS samples, integrated with inline analysis with tandem MS. This technique has the potential to reduce analysis time, increase throughput, and provide a flexible, re-configurable platform for the bioanalysis of pharmaceuticals in complex samples. Furthermore, this approach offers the possibility of changing the paradigm of how samples are analyzed away from centralized laboratories, and instead to facilities localized in closer proximity to the patient.

The incorporation of DBS into the workflow of pharmaceutical laboratories will depend on the application – high-throughput screening is likely best suited to robotics, while one-off or two-off analyses may be a good fit for DMF. Likewise, the widespread adoption of DMF as a platform to analyze DBS samples will depend on the capacity of the field to overcome several limitations and challenges. For example, the complex fabrication processes for each device and the lack of access to multiplexed droplet control systems are major hurdles to overcome if the widespread acceptance of

Table 2. Results of blind QC experiment.

Method	Parameter	Sitamaquine	Proguanil	Benzethonium chloride	Ibuprofen
–	[Actual drug] ng/ml	900	450	1000	200,000
Conventional	[Drug] ng/ml	978	468	1120	197,000
Conventional	Accuracy (%)	109	104	112	98.8
Conventional	CV of blind (%)	3.2	2.4	3.1	5.7
Conventional	Number of replicates	6	6	6	6
DMF	[Drug] ng/ml	814	457	669	240000
DMF	Accuracy (%)	90	101	67	120
DMF	CV of blind (%)	2.4	16	16	29
DMF	Number of replicates	3	4	8	8

DMF: Digital microfluidics.

this technology is to ever be reached. Open access automation systems such as DropBot [52] and the development of low-cost and resource-limited fabrication methods [53,54] may prove integral to the widespread adoption of DMF in coming years.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Device & test candidates

- A new digital microfluidic device was designed, consisting of four independent extraction zones, enabling the processing of four different DBS samples in parallel.
- A panel of six drugs were selected to test the new digital microfluidic extraction system, including sitamaquine, proguanil, benzethonium chloride, ibuprofen, acetaminophen and ibuprofen.

MS/MS analysis & optimization

- Sitamaquine, proguanil, benzethonium chloride and ibuprofen were successfully extracted and analyzed using digital microfluidic (DMF)-MS/MS, and were used to evaluate the new system.
- The total time required to extract and analyze a single DBS sample is approximately 6.5 min, representing a tenfold improvement relative to the standard method used in the industry.

Quantitative analysis

- Calibration curves were constructed for sitamaquine, proguanil, benzethonium chloride and ibuprofen for the purposes of quantitation.
- Replicate blind QC DBS samples were analyzed using the DMF protocol and compared with the traditional macroscale protocol. The DMF protocol performed similarly to the traditional protocol for sitamaquine and proguanil, but was less accurate than the traditional protocol for benzethonium chloride and ibuprofen.

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