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Mass Spectrometry & Spectroscopy

Automated Liquid Extraction Surface Analysis (LESA) from a Dried Blood Spot Card Holder via Chip-Based Nanoelectrospray

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This report describes a novel dried blood spot (DBS) card holder equipped with four defined surface extraction areas suitable for automated liquid extraction surface analysis (LESA) of dried biological sample spots. In brief, a commercially available DBS card containing four spotted whole blood samples is manually inserted into the card holder while a hinged cover containing a sequence of four circular ridges is clamped directly onto the sample substrate surface to define four constrained surface extraction regions for each sample. The LESA technique is then implemented by sequentially delivering a pipette tip containing a sample extraction/electrospray solvent to the sample surface. Analytes extracted from the DBS sample are then analysed directly by infusion nanoelectrospray mass spectrometry. Amlodipine and its tetradeuterated internal standard produced a linear calibration curve from 10 ng/mL to 10,000 ng/mL from fortified human whole blood with acceptable accuracy and precision using this approach.

Electrospray as an atmospheric pressure ionization technique has become a significant contribution to analytical chemistry when coupled with modern mass spectrometry. Although an early report of electrospray determination of industrial and biopolymers is what originally generated considerable interest in the technique [1] [2], it was the on-line coupling of HPLC with electrospray mass spectrometry (LC/MS) [3] as well as tandem mass spectrometry (LC/MS/MS) [4] that really propelled the interest and commercial development of this technology [5].

As a result we are all indebted to the late Professor John Fenn for his major contributions that have lead to an exciting addition to our analytical tool box. There are now thousands of publications describing a wide variety of impressive applications ranging from the qualitative characterisation of trace unknown, previously intractable, compounds to the widespread selected reaction monitoring (SRM) LC/MS determination of multi-residue substances in biological, environmental, and industrial samples.

In this report we describe an important modern extension of electrospray mass spectrometry employing microfluidics and chip-based devices that employ nano electrospray flows more than 1000-times lower than conventional electrospray procedures. This technology could potentially lend itself to lab-on-a-chip (LOC) devices in the future [6] as well as some noteworthy analytical benefits including improved analyte sensitivity, normalised response of related analytes and reduced ion suppression by matrix interferences [7]. In some instances, although counter intuitive at first thought, it is possible to analyse biological extracts by nanoESI via simple infusion sample introduction in lieu of HPLC sample separation [8]. This approach can lead to faster sample throughput in the absence of the often more time-consuming HPLC chromatographic separation process.

As an example of this latter concept we describe an automated procedure for the on-line sequential extraction of dried blood spots (DBS) coupled with chip-based infusion nanoESI analysis of the DBS extract. The subject of DBS, or dried 'matrix' spots (DXS) with 'X' being any sample matrix which is relevant to this format, is a topic of considerable current interest [9]. Traditionally DBS samples are used, for example, in newborn screening [10] and more recently in preclinical pharmaceutical drug discovery [9].

The strategy described in this report employs a robotic platform directly coupled to any modern atmospheric pressure ionisation (API) mass spectrometer system. The TriVersa NanoMate robot houses an ANSI (American National Standards Institute) format plate, which can accommodate a DBS card containing applied biological samples. The latter can range from whole blood, as described in this report, to plasma, urine, bile or a wide variety of other biological samples. The robot is equipped with a mandrel that can pick up a pipette tip containing a few microlitres of extraction/spray solvent, which extracts analytes from the sample surface via a micro liquid junction between the pipette tip and the DBS surface. By a repetitive dispense-aspirate procedure drug and metabolite residues contained on the DBS card surface are extracted into the pipette tip, which is then delivered to a microfabricated chip. At this point nanoESI commences to provide infusion electrospray mass spectra for the components extracted from the DBS card. This process is called liquid extraction surface analysis (LESA) and was first described by Kertesz et al. [11] [12] and more recently by Hooper et al. [13].

Here, we describe a semi-automated approach for a simple and effective way to automate the bioanalytical determination of drugs in DBS samples without the need to punch spots from the DBS substrate or application of surface tension modifiers. This work was first reported at the 58th Conference on Mass Spectrometry and Allied Topics [14].

Materials & Methods

DBS sample preparation

Control human, lithium heparin, whole blood (Bioreclamation, NY) was fortified with amlodipine across the concentration range from 10-10,000 ng/mL along with its tetra deuterated stable isotope internal standard fortified at the 4000 ng/mL level in each sample. Amlodipine was purchased as its besylate salt and reference standard (US Pharmacopeia, MD) and amlodipine-D4 was purchased as is maleic acid partner (Toronto Research Chemicals, ON). The chemical structures for these compounds are shown in *Figure 1*. Stock solutions (1 mg/mL) were prepared in 50/50 (v/v) methanol/water. The whole blood was mixed with the analyte stock solutions prior to spotting the paper blue striped FTA DMPK-C substrate cards (GE Healthcare/Whatman, NJ) to obtain the required amlodipine (10 ng/mL to 10,000 ng/mL) and D4 amlodipine (4000 ng/mL) concentrations. Aliquots (12 microlitres) of the fortified human whole blood samples were manually spotted onto paper cards via a pipette and air dried for 2h at room temperature.

Figure 1. Structures of amlodipine and its tetradeuterated internal standard

DBS Card Holder

Since it can be difficult to form a micro liquid junction between the pipette tip and a porous surface such as a cellulose-based DBS card, a special DBS card holder was fabricated in-house, from aluminum, to provide a means for solvent extraction of the DBS card from a defined region of the DBS sample. This was accomplished by installing four stainless steel (or peek) fittings (Idex, Inc, Bristol, CT 06010) with an inside diameter of 3mm in the hinged cover of the card holder and spaced to be centered over each of four dried blood spot sample locations. When the cover of the DBS card holder is lowered onto the base structure, the latter has a ridged surface to accommodate the rim of the mating fitting thus creating a tight, sealed region to confine dispensed extraction solvent applied to the DBS card surface *(Figure 2)*.

The card may be placed between the base and cover of this device such that the standard four circled regions, containing dried blood spot samples, are centered with respect to the fittings *(Figure 3)*. The hinged cover is then closed and clamped tightly onto the four DBS sample areas. As suggested in *Figure 2* the rim of the fittings create a sealed region in the centre of the applied dried blood spots which confines the solvent extraction region to a defined area of about 3mm diameter (vide infra). This allows the selected extraction-spray solvent introduced by the pipette tip to be distributed to a confined cross sectional area of the dried blood spot. This area is created by the perimeter of the fitting as it is compressed upon the dried blood spot sample.

The extraction-spray solvent for this application was 70% methanol, 30% water, 0.1% formic acid. It should be noted that the selection of this solvent should be predicated by a combination of appropriate solvent strength for optimal extraction of the target analytes at the expense of endogenous components as well as the appropriate nanoESI spray characteristics.

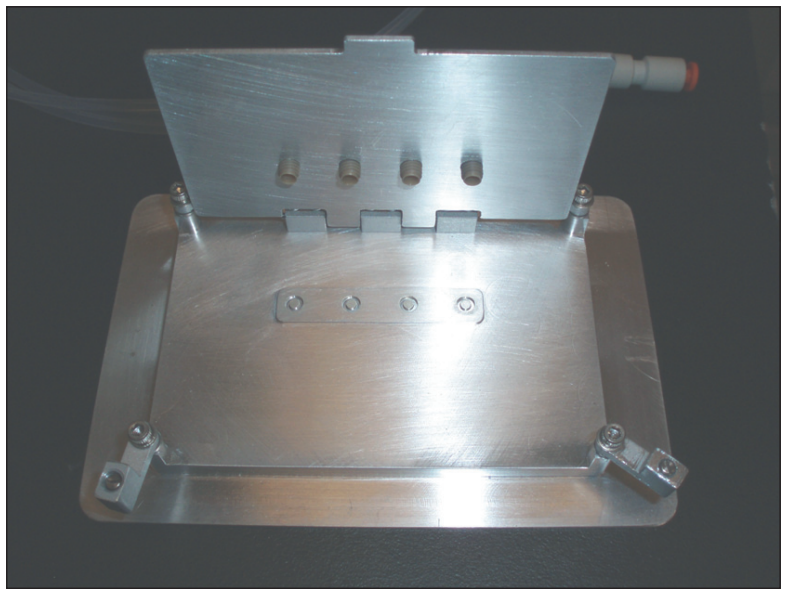

Figure 2. Photograph of DBS card holder showing the four PEEK ferrules which form a boundary region when the cover plate is closed tightly onto a DBS card.

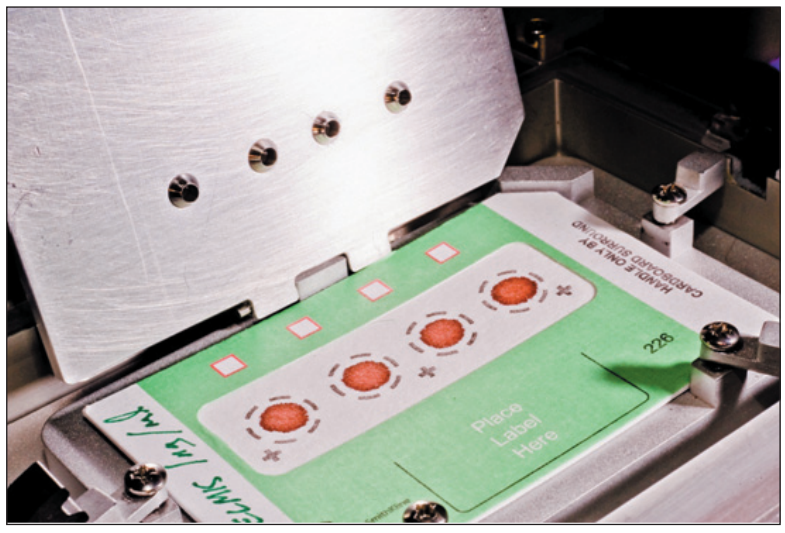

Figure 3. Photograph of a DBS card containing applied spots of whole blood. In this version stainless steel ferrules are employed to form the boundary region on the DBS card when the cover is clamped closed.

Mass spectrometry experimental conditions

An AB Sciex 4000 QTRAP tandem triple quadrupole mass spectrometer (Concord, Ontario,Canada L4K 4V8) was equipped with a TriVersa NanoMate (Advion BioSciences, Inc, Ithaca, NY 14850), which had been upgraded to LESA capabilities. The upgrade involved software changes that allow the conventional disposable pipette tip to access a special solvent reservoir affixed at one end of the platform. The DBS card holder described above had the same footprint as an ANSI 96-well plate so it could be placed directly into the sample holder of the platform stage of the system. The X,Y,Z coordinates for the four ferrule regions of the card holder were programmed into the TriVersa NanoMate to allow sequential sampling access to each dried blood spot sample. The 4000 QTRAP mass spectrometer was operated in positive ion nanoelectrospray SRM mode (approximately 200 nL/min infusion flow rate and the DBS sample typically extracted in 7 microliters of solvent). The transitions monitored included m/z 409.2 > m/z 237.9 for amlodipine and m/z 413.3 > m/z 238.2 for D4 amlodipine, respectively, with a dwell time of 50 msec. for each transition and employing nitrogen as the collision gas.

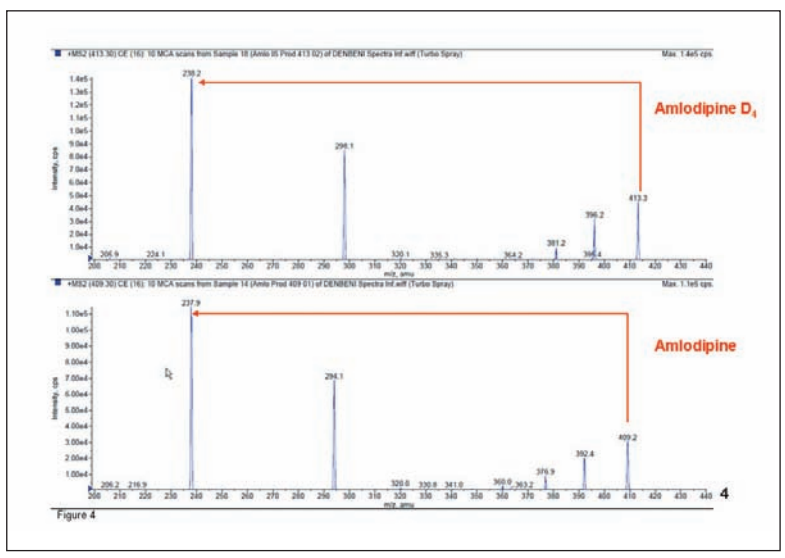

Figure 4. Collision-induced dissociation mass spectra for tetradeuterated amolipine (upper panel) and amlodipine (lower panel).

These transitions were selected from the characteristic fragmentation behaviour observed from full-scan CID mass spectra from these target compounds *(Figure 4)*. The infusion ion current signal area ratios between amlodipine and its D4 internal standard were plotted against the concentration of the standards. Typically the data acquisition for each sample was for 30 seconds and a 20 second portion of the ion current from the analyte and the internal standard, for each sample, were calculated as a ratio and plotted against the concentration for each standard and QC sample. The TriVersa NanoMate spray voltage was 1.65 kV with a head pressure of 0.3 psi on the sample extract contained within the pipette tip.

Discussion

Infusion nanoESI analysis of amlodipine in fortified dried blood spots.

Figure 4 shows the full-scan collision-induced dissociation (CID) mass spectrum for D4 amlodipine *(Figure 4a)* and amlodipine *(Figure 4b)*. These data were obtained via infusion nanoESI analysis of analytical standards of each compound using an AB SCIEX 4000 QTRAP mass spectrometer operated in the positive ion mode. In these infusion experiments, unlike LC/MS analyses, the analyte ion current signal is constant and allows signal averaging which can be beneficial when very low levels of target analytes are present.

The individual steps involved in using the TriVersa NanoMate platform for liquid extraction-surface sampling and subsequent infusion nanoESI-MS have been recently described in the literature [11]. In general, this includes an initial step where the robotic arm picks up a conductive pipette tip and moves the tip to the extraction solvent reservoir in order to pick up a 1-10 microliter aliquot of the extraction/spray solvent. Then, the pipette tip is positioned above the surface to form a micro liquid droplet maintained between the pipette tip and the sample surface. A sequence of dispense-aspirate steps affects an extraction of the sample surface whereupon the pipette tip is moved by the robot to the inlet of the microfabricated ESI chip and infusion nanoESI mass spectral acquisition is achieved employing either positive or negative ion detection.

Essential to the above approach of liquid extraction surface analysis is the formation of the micro liquid droplet maintained between the pipette tip and the sample surface such as a dried blood spot (DBS).

If the sample surface is highly absorptive, the liquid disperses away from the pipette tip and cannot be retrieved for subsequent infusion mass spectrometry. The purpose of the DBS sample card holder described in this report (Figure 2) is to create a defined region formed by the perimeter of the tapered stainless steel or PEEK ferrules to allow confinement and recovery of the extract.

Introduction of the extraction/spray solvent into the DBS card holder reservoir *(Figure 5a)* produces a small pool of solvent which is intended to extract sample of interest contained on the surface *(Figure 5b)*.

The diameter of the DBS surface extraction region in this work was 3mm and defined by the perimeter of the ferrules located in the hinged cover of the DBS card holder.

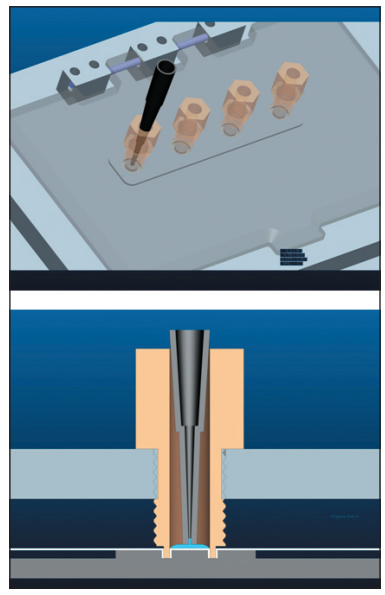

Figure 5a). CAD drawing showing a pipette tip inserted into a PEEK ferrule of the DBS card holder to extract the first dried spot sample.

b). Cross section CAD drawing showing the pipette tip inserted into the PEEK ferrule to a location just above the surface of the DBS spot while dispensing the extraction solvent to the confined region within the perimeter of the PEEK ferrule.

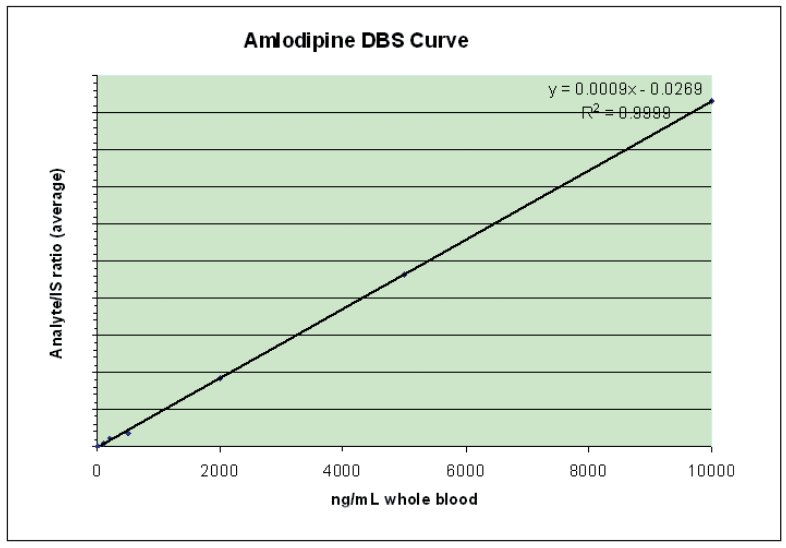

Figure 6. Calibration curve for the infusion SRM MS analysis of fortified whole blood samples using the device shown in Figure 5AB ranging from10 ng/mL to 10,000 ng/mL.

In the final step of the DBS sampling process, the sample extract obtained from *Figure 5b* is aspirated into the pipette tip and transferred to the inlet of the microfabricated ESI chip in the same manner as described above. This extract is then sprayed through a nanospray nozzle in the ESI chip while collecting the mass spectrometric response of the analyte of interest using either full-scan mass spectral acquisition or selected reaction monitoring (SRM) procedures for quantitative analysis applications. In the present version each of the four sample spots on the card may be robotically sampled and analysed followed by manual removal of the card from the card holder and placement of the next card into the holder for its similar analysis. This strategy will be useful when separate time points are collected on adjacent card spots. It should be noted that this DBS approach involves no punching of individual spots which is customarily described in the literature [9, 15], nor does it require additional card treatment to alter the surface hydrophobicity.

Results

To demonstrate proof-of-principle for the DBS card holder/clamp described above human Li heparin whole blood samples were fortified with amlodipine across a standard curve concentration range of 10 ng/mL to 10,000 ng/mL. Duplicate 15 microliter samples at two different concentrations were manually dispensed via a 25 microlitre syringe onto Whatman blue 'DMPK' paper cards. As an example, standard one was dispensed in the centre of the first two circled regions on the DBS card while standard two was dispensed in duplicate within the second two circled regions of the same DBS card. Thus a set of four DBS cards contained the first set of eight standards for the calibration curve. A second set of four identical cards were prepared with these eight standards to allow for the acquisition of a duplicate set of standard curve entries.

Figure 6 shows the calibration curve for the quantitative determination of amlodipine in fortified human whole blood using tetradeuteriated amlodipone as the internal standard. The dynamic range was linear from 10 ng/mL to 10,000 ng/mL amlodipine with a correlation coefficient of 0.9999. Since this work was exploratory, for proof-of-principle at this early stage, the additional studies common to regulated bioanalysis such as recovery, stability, and reproducibility, were not undertaken in this study.

Conclusions

The card holder described in this report provides a confined sample extraction region located within the sample spot for a dried blood or similar biological matrix. The described approach does not depend upon the porosity of the paper or other substrate upon which the sample matrix is applied. The LESA sampling pipette allows micro liquid extraction of a 3mm area of the sample from a porous substrate followed by direct infusion nanoelectrospray mass spectrometric analysis. Inter sample extraction and mass spectrometric analysis of the four spots contained on a given sample card is fully automated.

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Landmark Imaging System Sale

Paraytec Ltd have sold their 50th ActiPix UV Imaging System. This milestone sale was achieved in a new applications area for Paraytec in the pharmaceutical industry. Paraytec's latest system, the SDI300 allows pharmaceutical formulators to directly image drugs dissolving from a tablet surface, to enable more complete understanding of the drug behaviour in the body. This 50th system was sold in the United States by Paraytec's distribution partner, Distek Inc, to a large pharmaceutical company in California. The addition of this system to their laboratory will enable the scientists to avoid pitfalls in the development process, by providing essential understanding of an API (Active Pharmaceutical Ingredient) at an earlier stage than possible with traditional dissolution techniques.

Jeff Seely, Global Manager, Sales, Marketing & Technical Service for Distek, Inc said of the SDI300 system: "The new dimension the SDI300 brings to pharmaceutical API testing further enhances our offering to our customers." Mark Vaux of Paraytec Ltd, commented: "The in-depth knowledge Distek has of the US pharmaceutical industry enables the rapid uptake of Paraytec's technology, as we move towards having multiple systems in the major pharmaceutical companies."

The SDI300 is designed to provide key drug dissolution information on the new generation of sparingly soluble drug types, which require a new approach in formulation science. Circle no. 48