Adherence would provide more benefits to people worldwide than the World Health Organization suggested that 'improving medication understated problems for the healthcare systems worldwide. In 2003 therefore medication adherence must be regarded as one of the most to medication include medicine wastage and extended patient illness improper use of medicines [8]. Other consequences of poor adherence unnecessary deaths in the EU and USA annually resulting from adherence to prescribed medication.

The selection of specific cardiovascular medication was informed by discussion with practicing clinicians and a survey of drug prescribing

An example of incorrect adherence were identified. For example, amlodipine, valsartan, doxasozin and simvastatin was demonstrated using the LC-HRMS method. Furthermore with confirmed adherence were used to validate the response from the system as were samples from volunteers receiving no medication. No false positives were observed and adherence assessment for bisoprolol, ramipril, amiodipine, valsartan, doxasozin and simvastatin was demonstrated using the LC-HRMS method. Furthermore examples of incorrect adherence were identified.

Keywords: Cardiovascular medications; LC-HRMS; LC-MS; Dried blood samples

Introduction

Liquid chromatography - high resolution mass spectrometry (LC-HRMS) analyses now rival LC-MS/MS techniques in terms of both compound specificity and quantitative detection capabilities [1–4]. LC-MS/MS techniques depend on the predetermination of the fragments ions from a precursor ion characteristic of the target analyte. For the drug captopril [5], for example, the precursor ion would be MH+ at m/z 218.0 and the previously identified fragment ions are m/z 172.0 and 116.0. These are the values applied to MS1 and MS2 in an LC-MS/MS (triple quadrupole) instrument in order to detect captopril during the elution of a sample. In the HRMS (Time of Flight MS) instrument all ions are recorded during sample elution and this data is then scanned for the presence of ions at m/z 218.0845 characteristic of captopril. In analyses of this type the ToF MS measures m/z values typically to an accuracy of 10 parts per million in mass. This approach means that the LC-HRMS results can be re-interrogated for other analytes at a later date without the need to re-run samples. In this paper LC-HRMS analyses of Dried Blood Spot (DBS) samples are used to assess patient adherence to prescribed medication.

Adherence to medication refers to whether a patient takes their medication exactly as prescribed by the clinician or doctor [6], for example, three times a day after meals or one to be taken at night. Furthermore the complete course of medication should be taken as required by the prescription. Despite what might be expected levels of adherence to medication are NOT good. Examples of poor adherence include ~40% for people with heart disease in the US and UK, ~ 50% for patients with breast cancer and as low as ~15% for some haematological cancers [7]. It is not surprising therefore that there are some 320,000 unnecessary deaths in the EU and USA annually resulting from improper use of medicines [8]. Other consequences of poor adherence to medication include medicine wastage and extended patient illness resulting in additional hospital and healthcare costs [9]. Clearly therefore medication adherence must be regarded as one of the most understated problems for the healthcare systems worldwide. In 2003 the World Health Organization suggested that ‘improving medication adherence would provide more benefits to people worldwide than the development of new medicines’ [10]. In the UK the medicines costs, within the NHS, constitute the second largest identifiable unit after staff costs [11]. Improved adherence to medication offers an opportunity to reduce or at least optimize some of the £11 billion current costs to the NHS.

Direct analysis of patient biofluids (blood, urine or saliva) provides the only unambiguous assessment of adherence to prescribed medication. Other recognized methods of assessment can produce misleading information, for example, tablets are collected but may not be taken, there are optimistic answers to questionnaires and patients may mislead clinicians [12]. Whilst both urine and saliva analyses can be used to show adherence the relationship between both the time of ingestion and the dose taken cannot readily be established. The work reported here demonstrates that the analyses of Dried Blood Spots (DBS) samples, collected from a minimally invasive finger prick, can provide a quantitative assessment of adherence to medication. The advantages of DBS based methods coupled with improved analytical instrumental capability has led to a surge in the use of this sampling methodology for various applications [13,14].

Experimental

Target cardiovascular (CV) medication

The selection of specific cardiovascular medication was informed by discussion with practicing clinicians and a survey of drug prescribing

Abstract

Research suggests that ~60% of patients prescribed cardiovascular drugs do not take their medication correctly. The analysis of dried blood samples (DBS) by liquid chromatography-high resolution mass spectrometry (LC-HRMS) for assessing medication adherence to candidate therapeutic drugs used in cardiovascular therapy was investigated. Specificity using this analytical method was based on the measurement at the accurate mass to charge ratio of the target analyte. To evaluate the method 8mm discs were punched from each DBS and extracted followed by subjecting to LC-HRMS analysis. Trials on 6 commonly UK used cardiovascular drugs are reported demonstrating the ability of the system to detect the target analytes during the 24 hour repeat prescription cycle. Samples from volunteers with confirmed adherence were used to validate the response from the system as were samples from volunteers receiving no medication. No false positives were observed and adherence assessment for bisoprolol, ramipril, amiodipine, valsartan, doxasozin and simvastatin was demonstrated using the LC-HRMS method. Furthermore examples of incorrect adherence were identified.
in the UK. The medicines identified as frequently prescribed CV drugs in the UK and selected for this study were:

- Bisoprolol  beta blocker
- Ramipril  ace inhibitor
- Simvastatin  statin
- Amlodipine  calcium channel blocker
- Doxazosin  alpha blocker
- Valsartan  angiotensin receptor blocker.

Table 1 details the relevant pharmacokinetic (PK) information which will influence the detection capabilities required for this investigation. The aim of this work was to investigate if the presence of specified target drugs can be confirmed in blood samples from individual volunteers and therefore the ranges in the responses from different volunteer/drug combinations is more important than a mean value. Specifically therefore the worst case scenario, i.e. the lowest likely drug concentration in the DBS sample, must be considered. This will occur for a combination of low bioavailability, low dose, rapid elimination (short t_{1/2}) with a low C_{max} developed in the shortest time (t_{max}). In this respect simvastatin should pose the major challenge. The other major factor affecting the level of drug to be detected was the time delay between the patient taking the medication and the subsequent provision of the blood spot sample.

The data in (Table 1) shows the drug concentration in blood rising to a maximum (C_{max}) usually after a short time (t_{max}) and then decreasing exponentially at a rate governed by t_{1/2}. This data is usually derived from experiments based on a single dose given to healthy volunteers. For long-term adherent volunteers a ‘steady state’ condition develops in which the initial rise in concentration is less marked and the rate of decrease is reduced. Under adherence conditions the steady state level, of the drug in blood, is the minimum level of drug available for detection. An indication of patient non-adherence will be derived from a combination of no signal above the instrument noise level and the passage of at least 5 half-lives abstinent.

**Volunteer samples**

In the initial phase of this investigation two sets of volunteers were selected from informed colleagues:

- A group who routinely took one or more of the target medicines
- A group who were prescribed none of the target medicines.

The volunteers in the first group, provided blood samples at specified times after taking known medication in order to provide controlled tests of the detection system. Analysis of DBS samples from the second group, volunteers known to take none of the target drugs, was used to provide baseline reference data for the trial samples.

| Drug      | Dose range/mg | Cmax /ng/ml | Tmax/h | Thalf/h |
|-----------|---------------|-------------|--------|---------|
| Bisoprolol [14] | 2.5/5/10*     | 37-87       | 1.5-4  | 5-16    |
| Ramipril  [15]  | 2.5/5/10      | 11-31       | 1-4    | 4-6     |
| Amlodipine [16-18] | 5/10         | 7-5         | 5-8    | 35-50   |
| Valsartan [19] | 40/80/160*   | 379-3874    | 2-8    | 3.5-14  |
| Doxazosin [20] | 1/2/4/8/6*   | 67/17.6     | 2.7-5  | 20.5 (6.1)|
| Simvastatin [21] | 10/20/40*   | 5-40        | 2-3    | 1.3-2.7 |
| Atenolol [14] | 25/50*       | 159-377     | 1.5-6.0| 4-11    |

Replicate (n=5) ~30 µl volume blood spot samples were collected on Whatmann 903 sample paper either in the laboratory or by volunteer self-collection at home. The delay time between taking the prescribed medication and DBS sample collection was noted. After collection the samples were allowed to dry under ambient conditions for a minimum of 2 hours and were then sealed into plastic bags containing a desiccant. Self-collection at home was seen as a move towards cost saving versus conventional liquid blood sample collection. Volunteers willing to try this approach attended a laboratory training session.

**Chemicals and materials**

Acetonitrile, methanol and water of LC-MS grade were purchased from Sigma-Aldrich (Poole, UK). The active pharmaceutical ingredients (API) Atenolol (R(-), 99%), bisoprolol fumarate salt, ramipril, valsartan, amlodipine besilate, simvastatin and doxazosin mesilate were also purchased from Sigma-Aldrich (Poole, UK). Formic acid, auto-sampler vials with 250 µl inserts and vial caps were purchased from Agilent Technologies (Cheshire, UK). Specimen collection paper type 903, micro-centrifuge tubes (1.5 ml), volumetric pipettes, pipette tips and polyethylene bags were obtained from Fisher Scientific (Loughborough, UK). An 8 mm diameter punch was obtained from Maun Industries Ltd. (Nottingham, UK). Lithium heparin coated blood collection tubes were purchased from International Scientifique Supplies Ltd. (Bradford, UK). Fresh blank blood was obtained from informed volunteers in line with De Montfort University Ethics Protocols.

**Sample analysis**

The preparation of standard solutions and spiked blood spot samples to produce calibration data for quantitative determinations has been detailed elsewhere [2] and is not duplicated here. An 8 mm disc was punched from the centre of the DBS sample and transferred to a 1.5 ml micro-centrifuge tube. A 150 µl of extraction solvent consisting of methanol/water (70:30, v/v) plus the internal standard (20 ng/ml) was added to this. Sample tubes were then vortexed for 1 min and sonicated for 30 min. They were then centrifuged for 10 min at 13.2 x g and each extract was transferred to an auto-sampler vial for analysis by LC-HRMS.

The LC-HRMS system consisted of an Agilent 1290 UPLC coupled to an Agilent 6530 QTOF mass spectrometer, used in the TOF only mode. The target drugs were analysed on an Zorbax Eclipse C18 rapid resolution HD column (Agilent Technologies, Cheshire, UK, 100 mm x 2.1 mm i.d., 1.8 µm particle pore size) which was preceded by a 0.3 µm inline filter (Agilent Technologies, Cheshire, UK). The oven temperature was set to 40°C. Sample injection volume was 20 µl. The mobile phase consisted of water containing 0.2% v/v formic acid (eluent A) and acetonitrile containing 0.2% v/v formic acid (eluent B) and was delivered at 0.6 ml/min with gradient elution. The mobile phase was initiated at 5% B and maintained for 0.5 min before increasing to 20% B and then to 95% B by 1.5 min and held until 3.0 min before returning to 5% B. The gradient elution programme was then held for 1.5 min to re-equilibrate the column prior to the next injection. The mass spectrometer was operated in the electrospray positive ion mode. The MS source and chamber conditions were optimised to give maximum analyte signal intensities as follows: Fragmentor voltage: 165V; Skimmer: 65V; Gas Temperature: 350°C; Dry Gas: 10 l/min; Nebuliser: 50.0 psi; Sheath Gas Temperature: 400°C; Sheath Gas Flow: 20 l/min. Mass Range: 100-1000 m/z; Recording Rate: 1 Hz. HRMS lock reference masses: 121.0508 m/z and 922.0098 m/z. Data acquisition was controlled by the Mass Hunter Workstation Software for TOF/Q-
TOF version B.04.00 (Agilent Technologies) and the acquired data was processed using Qualitative Analysis B.04.00 and Quantitative Analysis B.04.00 software (Agilent Technologies). External calibration of the TOF mass spectrometer was performed daily prior to starting the analysis.

Validation
A validation of this approach, following international guidelines, including data on selectivity, linearity, sensitivity, intra and inter-assay accuracy and precision has already been published [2].

Results and Discussion

Selectivity
Before any analyses could be undertaken it was necessary to determine the accurate masses of the target analytes and internal standard. Standard mass spectra in the full scan range 100-1000 m/z were obtained by injection of a multicomponent standard solution of the target drugs and the internal standard (20 ng/ml) in a mixture of methanol and water (70:30, v/v). The most intense ions under the chosen operating conditions were:

| Drug       | m/z       | Species   |
|------------|-----------|-----------|
| Bisoprolol | 326.2326  | MH+       |
| Ramipril   | 417.2384  | MH+       |
| Simvastatin| 441.2611  | MNa+      |
| Amlodipine | 431.1344  | MNa+      |
| Doxazosin  | 452.1928  | MH+       |
| Valsartan  | 436.2343  | MH+       |
| Atenolol (IS) | 267.1703 | MH+       |

Figure 1 shows the Extracted Ion Chromatograms (EICs) for these compounds using the appropriate accurate masses. These traces show that there were no interferences at the same retention time of each of the drugs which confirmed the good selectivity of the method.

Volunteer samples
To date a total of 488 samples of different combinations of volunteer/target drug/time delay after dosing have been received from the volunteers taking medication including 40 samples from volunteers not prescribed any of the medicines investigated in this project. Analytical investigations routinely involve blank samples, baseline samples, calibration samples and the test samples. In this investigation blank samples were those provided by the volunteers known not to be taking any of the subject medicines. Baseline samples were obtained from volunteers immediately prior to them taking the due dose of medication. These samples corresponded to delays of at least 24 hours and demonstrating a detection capability at this level eliminates the possibility of reporting false negatives. A false negative output would occur if the system was unable to detect the real levels of the target drug present for an adherent volunteer (Figure 1).

Sample Quality: In order to provide useable data the blood sample must be uniform and of sufficient size to provide an 8mm diameter punched disc. All the laboratory collected samples met these requirements but 26 of 135 DBS samples collected at home were unsatisfactory. The spots on the unsatisfactory samples were either too small or smeared which meant the blood sample was not uniform. This represented a 19% failure rate for home sample collection.

Sample time delay distribution: As might be expected, assuming most volunteers take their medication in the morning, the majority of the samples were collected within 12 hours of the dose being taken. More than 60% of the samples collected were within this timeframe and 26% had delay times between 12 and 18 hours. The delay time for simvastatin medication, taken at night, was either less than 4 hours (50% of samples) or in excess of 16 hours (40% of samples).
corresponding to an overnight delay period. Periods in excess of 24 hours were derived from judicious delays in taking the following dose. These results are summarized in Table 2. The total success of this phase of the investigation is testament to both the resolute adherence of the volunteers and to the performance of the system under this mode of operation. Inspection of the data for periods of >24 hours would suggest that whilst ions appropriate to simvastatin were detectable the levels seen would have been below the limits of quantification.

### Qualitative assessment of adherence

All data was processed as the ratio of the peak area for the particular accurate mass divided by the peak area for the internal standard from the same sample extract. This approach was designed to reduce variations in the very low levels detected, resulting from minor differences in extraction efficiency and the analytical conditions. The baseline samples, i.e. those with a delay between 24 and 25 hours all showed a detectable signal for all the relevant target API for those volunteers who were known to be taking the specified medicines. This would be expected for regular medicine users who would develop a ‘steady state’ level of the target drug in the blood. By contrast analysis of the samples from volunteers not taking any of the medicines (blank samples) produced no signal greater than the noise level at the retention times identified to be of interest. This observation confirms both the performance of the approach i.e. identifying absence of the target drugs and also that there were no other species present that could lead to an interfering signal. All of the samples from the control adherents to medication showed the presence of the expected target drugs which may either confirm good adherence or that knowing a test is coming will ensure the appropriate action, taking the medicine, occurs.

### Quantitative detection of the target cardiovascular drugs

The quantitative work that has been carried out has been reported elsewhere [2] and has been validated in line with International guide lines on Bioanalytical method validation as summarized below:

- Specificity-good for all the APIs investigated
- Variation in accuracy and precision less than 15%
- Linearity better than 0.991
- Determination of LoD typically better than 1.0 ng/ml
- Recovery range from ~98% for bisoprolol to ~40% for simvastatin
- Matrix effects < 15%
- Samples stable for 12 weeks.

Quantitative information not only confirms that the medicine was taken but may also offer a further degree of assessment of precise adherence to prescription instructions. The concentrations of the target CV drugs determined in this work should relate to the data in Table 1 which cites literature information on the maximum concentrations ($C_{\text{max}}$) of these target drugs in the blood. The quantitative results were examined and in the measured concentrations were all of the same order as or below the $C_{\text{max}}$ levels from Table 1. In one set of replicates the level determined for the simvastatin concentration was 50% higher than the maximum expected. This high level raised a question since it is known that eating grapefruit products when taking simvastatin should be stopped. Under normal conditions the bioavailability of simvastatin is only ~5% with the remainder being metabolised before it can be absorbed into the blood. Grapefruit products compete with this metabolic pathway and consequently the levels of simvastatin in the blood can rise significantly [21]. When questioned, the volunteer explained that he took all his pills at the same time so he did not forget the simvastatin which should be taken at night. There was no evidence of grapefruit in the diet but one of his other CV drugs was known to have a similar effect on the metabolism of simvastatin. Here we have an example of the ability to identify non-adherence to prescription instructions, resulting in a medication error, which could only be identified from quantitative data.

### Conclusion

The advantages of using LC-HRMS analyses of the target CV drugs in DBS samples have been demonstrated for assessing medication adherence. The developed DBS based method correctly assessed adherence in a non-clinical setting and furthermore samples collected at home provided a viable but limited route to valuable data collection. Qualitative data provides a quick but limited assessment of adherence whereas quantitative data enabled a less obvious form of non-adherence to be identified. It is essential to ensure that the limit of detection for each target CV drug is below the steady state level for adherent volunteers.

### References

1. Lawson G, Cocks E, Tanna S (2012) Quantitative determination of atenolol in dried blood spot samples by LC-HRMS: a potential method for assessing medication adherence. See comment in PubMed Commons below J Chromatogr B Analyt Technol Biomed Sci 897: 72-79.
2. Lawson G, Cocks E, Tanna S (2013) Bisoprolol, ramipril and simvastatin determination in dried blood spot samples using LC-HRMS for assessing medication adherence. See comment in PubMed Commons below J Pharm Biomed Anal 81-82: 99-107.
3. Zhang T, Watson DG, Azike G, Teltley JN, Stearns AT, et al. (2007) Determination of vancomycin in serum by liquid chromatography-high resolution full scan mass spectrometry. See comment in PubMed Commons below J Chromatogr B Analyt Technol Biomed Sci 857: 352-356.
4. Jiwan JL, Wallemacq P, Hérent MF (2011) HPLC-high resolution mass spectrometry in clinical laboratory? See comment in PubMed Commons below Clin Biochem 44: 136-147.
5. Lawson G, Mulla H, Tanna S (2012) Captopril determination in dried blood spot samples with LC-MS and LC-HRMS: A potential method for neonate pharmacokinetic studies. J Bioanal Anal 4: 016-025.
6. Anon (2012) Medication adherence. Patient Resource Cancer Guide.
7. Tanna S, Lawson G (2014) Dried blood spot analysis to assess medication adherence and to inform personalization of treatment. See comment in PubMed Commons below Bioanalysis 6: 2825-2838.
8. Anon (2008) PEGU GPUTE Policy Statement - Targeting adherence. Brussels.
9. Jackevicius CA, Li P, Tu Jv (2008) Prevalence, predictors, and outcomes of primary nonadherence after acute myocardial infarction. See comment in PubMed Commons below Circulation 117: 1028-1036.
10. WHO (2003) Adherence to long-term therapies: Evidence for action. (1st edition) Sabete E, ed. Geneva, Switzerland: WHO Publications 211.
11. http://www.nhsconfed.org/resources/key-statistics-on-the-nhs Accessed 16 October 2014.pdf
12. Ruddy K, Mayer E, Partridge A (2009) Patient adherence and persistence...
with oral anticancer treatment. See comment in PubMed Commons below CA Cancer J Clin 59: 56-66.

13. Tanna S, Lawson G (2011) Analytical methods used in conjunction with dried blood spots. Anal Methods 3: 1709-1718.

14. Lewis R, Maclean D, Ioannides C, Johnston A, McDevitt DG (1988) A comparison of bisoprolol and atenolol in the treatment of mild to moderate hypertension. See comment in PubMed Commons below Br J Clin Pharmacol 26: 53-59.

15. Hosie J, Meredith P (1991) The pharmacokinetics of ramipril in a group of ten elderly patients with essential hypertension. See comment in PubMed Commons below J Cardiovasc Pharmacol 18 Suppl 2: S125-127.

16. Shah SK, Asnani AJ, Kawade DP, Dangre SC, Arora SK, et al. (2012) Simultaneous quantitative analysis of olmesartan, medoxomil and amlodipine besylate in plasma by high performance liquid chromatography technique. J Young Pharm 4: 88-94.

17. Vincent J, Harris SI, Foulds G, Dogolo LC, Willavize S, et al. (2000) Lack of effect of grapefruit juice on the pharmacokinetics and pharmacodynamics of amlodipine. See comment in PubMed Commons below Br J Clin Pharmacol 50: 455-463.

18. Meredith PA, Elliott HL (1992) Clinical pharmacokinetics of amlodipine. See comment in PubMed Commons below Clin Pharmacokinet 22: 22-31.

19. Zaid AN, Cortesi R, Qaddomi A, Khammash S (2011) Formulation and bioequivalence of two valsartan tablets after a single oral administration. See comment in PubMed Commons below Sci Pharm 79: 123-135.

20. Chung M, Vashi V, Puente J, Sweeney M, Meredith P (1999) Clinical pharmacokinetics of doxazosin in a controlled-release gastrointestinal therapeutic system (GITS) formulation. See comment in PubMed Commons below Br J Clin Pharmacol 48: 678-687.

21. Lilja JJ, Neuvonen M, Neuvonen PJ (2004) Effects of regular consumption of grapefruit juice on the pharmacokinetics of simvastatin. See comment in PubMed Commons below Br J Clin Pharmacol 58: 56-60.