The Effect of Sample Tube Type, pH, Storage Time and Temperature on Antihypertensive Non-Adherence Results by Quantitative LC-MS/MS

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Introduction

Hypertension is a global health epidemic, and is the leading cause of disease morbidity and mortality in the world. It can be treated with medication. Non-adherence to hypertension medication has been found to be a common problem; around 25% of patients show some degree of non-adherence. Biochemical testing has been developed as a direct and objective measure to diagnose non-adherent behaviour. These are qualitative screens that use liquid chromatography-tandem mass spectrometry (LC-MS/MS).

The effects of pre-analytical factors have been suggested to lead to false negative results and have not yet been fully explored. For example, the type of sample tubes used for urine collection, as well as the storage temperature of samples prior to analysis, have been cited as possible causes of false negative results in non-adherence screening. Urinary pH is considered useful to test the integrity of urine samples in toxicology, with pH levels outside of pH4-9 also leading to possible false negative results.

Aim

The aim of this study was to investigate the impact of these pre-analytical factors on detection of antihypertensive drugs in urine, studying: the effects of different sample tubes routinely used for sample collection, urine pH and urine storage temperature.

Method: *Validation*

A quantification assay was developed for six antihypertensive drugs and were validated using the Waters Acquity XEVO TQD LCMS system with an ESI Z-spray triple quadrupole mass spectrometer in positive ion mode. The mass spectrometry parameters have been summarised in Table 1.

Drug	Precursor Ion (m/z)	Product Ions (m/z)	Cone Voltage (V)	Collision energy (V)	Retention time
Amlodipine	409.18	237.95	18	16	5.01
Amlodipine-d4	413.27	238.05	18	14	5.01
Atenolol	267.19	145.05	52	26	1.35
Atenolol-d7	274.22	145.12	38	26	1.35
Indapamide	366.09	131.95	24	14	5.03
Indapamide-d4	369.12	135.08	26	20	5.03
Losartan	423.19	207.06	32	24	5.30
Losartan-d4	427.27	211.02	32	20	5.30
Propranolol	260.13	116.01	4	18	3.99
Propranolol-d7	267.23	116.42	36	20	3.99
Verapamil	455.32	165.02	76	28	5.01
Verapamil-d7	462.29	165.08	46	26	5.01

Table 1. Mass Spectrometry parameters for each analyte in the LC-MS/MS method

The validation was carried out according to the Center for Drug Evaluation and Research (CDER) Guidance for Industry: Bioanalytical Method Evaluation, 2001 and the following parameters assessed were: precision, accuracy and recovery, linearity, carryover, selectivity, matrix effect, calibration curve, lower limit of detection (LLOD) and lower limit of quantification (LLOQ).

Sample analysis

Urine sample, calibration standard or QC (100 µL) was vortexed with IS (25 µL) and deionised water (875 µL).

Calibration curve

Seven calibration standards were prepared in pooled blank urine at: 25, 50, 100,125, 250 and 1000 ng/mL. Quality control samples (QC's) were prepared at 30 (low), 50 (mid) and 800 (high) ng/mL. A mixed internal standard containing all six analytes was prepared at 200 ng/mL.

LLOQ

LLOQ was defined as the lowest calibration standard: 25 ng/mL. It should also meet the accepted criteria for bias and precision.

Carryover

This was measured on three separate occasions by injecting two of the highest standards (1000 ng/mL) followed by two extracted blank samples containing no IS. Carryover was deemed insignificant if the response for the analyte and IS was less than that observed at the LLLOQ.

Selectivity

Six independently sourced patient urine samples were analysed. The lack of peaks at the retention time for each analyte would indicate acceptable selectivity with a lack of interfering substances from the matrix and all results should be less than the LLOQ.

Matrix effects

The analyte and IS response for post-extracted spiked samples were compared to post-spike solvent samples. The % matrix effects were calculated= [(response post extracted spike sample/ response post-spike solvent samples)-1] x 100. The matrix effects were deemed insignificant if <±20%.

Accuracy, precision and recovery

QCs were analysed in triplicate over five batches and LLOQ analysed five times in once batch. Precision was sub-divided into within-run and intra-run precision. Accuracy was deemed acceptable if the mean value was within 15% for the QCs and 20% at the LLOQ of the actual value. Precision was deemed acceptable if the within-run and between run %CV were <15% for the QCs and <20% for the LLOQ.

LLOD

LLOD was deemed acceptable if <20% CV of the mean concentration of a minimum of three samples per run over five runs.

Method: Stability Study

The effects of storing urine samples in various sample tubes at pH 4,6 and 8 at room temperature and at refrigerated temperature (2-8˚C), for three days and seven days was investigated.

Sample tube types

The sample tubes assessed are shown in Figure 1 A-F.

Figure 1. A photograph of the urine sample containers investigated in this study. A: glass; B: monovette; C: vacutest 60 mL; D: vacutest 9 mL; E: universal; F: metal cap.

Results: *Validation*

All validation criteria defined by Centre for Drug Evaluation and Research (CDER) were met for each of the drugs.

Carryover

No significant carry over was observed up to 1000 ng/mL.

Selectivity

No significant interference from the 6 blank matrix samples analysed. The LLOQ acceptance criteria were ±20% bias against the nominal concentrations and ≤ 20 CV%.

LLOD

LLOD for each drug was below 10 ng/mL with CV% <20.

Accuracy, Precision and Recovery

For each of the analytes for within run precision and between run precision CV% <10 and bias % within run and between run <15.

Results: *Statbility Study*

Figure 2 shows the % recoveries for each drug at the varying pH values, temperature, sample tube types and storage time. Amlodipine, atenolol and verapamil stabilities were most affected by pre-analytical factors. Overall recoveries ranged from 56.1% to 102.2%. The recoveries of all six drugs significantly decreased from pH 4 to pH 8. Stability at room temperature was higher compared to 4˚C. There was a small drop in recoveries of amlodipine, indapamide and verapamil at seven days vs. three days. Glass containers appear to least effect the sample stability while it was the worst with metal cap containers. Universals also had poor recoveries for each of the drugs.

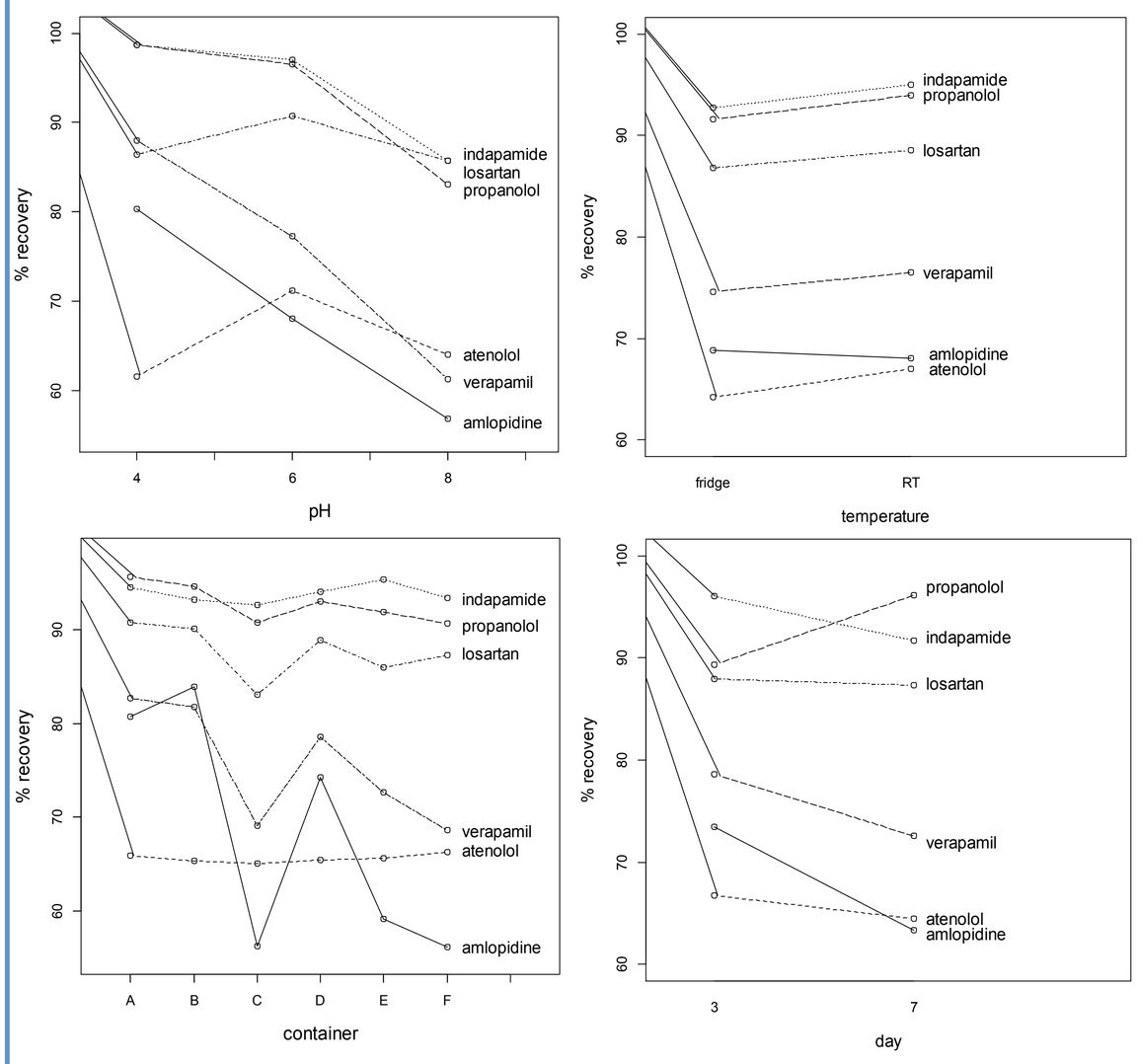

Figure 2. Overall mean %recovery for each drug for A: pH 4, 6, and 8; B: fridge and room temperature; C: containers A-F; and D: day 3 and 7

Discussion / Conclusion

Quantitative LC-MS/MS shows there is an effect of pre-analytical factors on levels of antihypertensive medication detected.

Temperature influenced atenolol and indapamide recoveries with lower % recoveries observed in the fridge compared to room temperature. The difference in % recoveries wasn't hugely significant however it did show that adherence or precipitation of certain drugs at lower temperature is plausible.

Drug stability decreased over time. Basic pH decreased stability, sample containers also effected stability.

Sample tube type also had a substantial influence on four out of six of the drugs. C (the Vacutest 60mL), E (Universal) and F (the metal cap**)** appear to have the lowest % recoveries. This was expected as the influence of sample tube plastic has previously been shown to promote the absorption of analytes onto the plastic containers.

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