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A Novel 'Dilute-and-Shoot' Liquid Chromatography–Tandem Mass Spectrometry Method for the Screening of Antihypertensive Drugs in Urine

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Abstract

Arterial hypertension is one of the most preventable causes of premature morbidity and mortality with resistant hypertension reported to be present in 5–30% of the total hypertensive population. Despite the poor prognosis, as many as 53% of those with resistant hypertension are reported to be nonadherent to their prescribed medication. An objective test of adherence, which is easy to administer, quick, inexpensive and reliable, is therefore needed to identify patients with true resistance to antihypertensive drugs to optimize their treatment. We have developed a novel LC–MS-MS method for the detection of 23 commonly prescribed antihypertensive medications in urine. The validated method was subsequently applied to the analysis of urine from a cohort of 49 individuals who were taking at least one antihypertensive agent in the screening profile to determine their adherence. The screening method was found to be reproducible, sensitive and specific with the limit of detection ranging from 0.1 to 1.0 µg/L. Sample preparation is rapid (30 s) and simple, with a total analysis time of 11 min. The assay successfully identified the majority of drugs our cohort had admitted to taking (88%) with drugs not detected in urine, potentially indicating nonadherence to prescribed medication. The performance of this simple, robust LC–MS-MS procedure is suitable for screening urine for the presence of commonly prescribed antihypertensive medications. The assay, which can easily be implemented in other laboratories, has the potential to significantly improve investigation and management of resistant hypertension.

Introduction

Arterial hypertension is one of the most preventable causes of premature morbidity and mortality in the world. The global prevalence of hypertension in adults was 26% in 2000 and is expected to go up to 29% in 2025 (1). It contributes to 62% of strokes and 49% of heart disease, leading to 7.1 million deaths a year, equivalent to 13% of all deaths in the world (2). Successful blood pressure (BP) lowering results in reductions of both morbidity and mortality (3).

Resistant hypertension is defined as BP above 140/90 mmHg while on ≥3 antihypertensive agents (one of which is usually a diuretic) at optimal or maximum tolerated doses (4–7). Patients with resistant hypertension are almost 50% more likely to experience a cardiovascular event compared with those without resistant hypertension (8). Resistant hypertension is reported to be present in 5–30% of the total hypertensive population (6). However, true prevalence of resistant hypertension is difficult to determine because of apparent resistance due to white coat hypertension, poor adherence with prescribed

medication, poor BP measurement technique and inappropriate combination of treatment (5).

Poor medication adherence is common among patients with hypertension, with many studies reporting low adherence rates associated with inadequate BP control (9–13). As many as half of the patients labeled as having resistant hypertension have been reported to be nonadherent to prescribed medication (14). Our own experience from a directly observed therapy (DOT) clinic is similar, with suboptimal medication adherence being present in as many as half of the patients with resistant hypertension (15).

Despite the recognition that nonadherence is an important public health problem, the definitions of adherence vary and tests for adherence are imperfect (16). A number of indirect methods are commonly used to assess adherence including patient interviewing, prescription refill and pill counts. In the UK, most centers admit patients with resistant hypertension to hospital for supervised administration of medications and monitoring of BP. A few centers have established DOT clinics (15, 17). This method is costly in regard to bed/clinic usage and staff time, and inconvenient to patients. Therefore, there is a need for the development of an objective test of adherence, which is easy to administer, quick, inexpensive and reliable with a view to improving patient care by identifying those with true resistance to antihypertensive treatment. This will also allow research into areas including the factors affecting and tools designed to improve medication adherence (16).

Numerous techniques have been employed by laboratories to screen various biological matrices for drugs and metabolites. These techniques have included immunoassay, thin layer chromatography (TLC) and gas or liquid chromatography with or without mass spectrometry. For many years, gas chromatography with mass spectrometry (GC–MS) was the method of choice for toxicological analysis and is still of great value in the analysis of certain analytes, e.g., toxic alcohols (18). Owing to the superior specificity and sensitivity offered by liquid chromatography–tandem mass spectrometry (LC–MS/MS) coupled with the relative ease of sample preparation when compared with GC–MS, has seen LC–MS/MS adopted in many clinical laboratories for a number of biochemical and toxicological applications (19–22). Samples for LC–MS/MS analysis may still require some degree of sample preparation before analysis [for example, protein precipitation, liquid–liquid extraction or solid-phase extraction (SPE)] depending on the characteristics and concentration of the analyte in question. Recent improvements in the sensitivity of LC–MS-MS systems have led to the development of screening techniques, which allow samples to be simply diluted and then directly injected onto the analytical system (23). This offers a rapid sample preparation with minimal staff time and reagent cost when compared with other techniques, such as SPE, and has the advantage of screening for the broadest range of compounds as no one class of compound (e.g., opioids) is targeted in the extraction (c.f. liquid–liquid extraction).

Here, we present a novel 'dilute-and-shoot' LC–MS-MS method for the detection of a broad range of antihypertensive medications in human urine and show that the method is suitable for use in the investigation of resistant hypertension.

Materials and methods

Drug calibrators and internal standard preparation

Stock solutions of all drugs (Table I) were prepared by dissolving pure drug (Sigma, Poole, UK) in HPLC-grade methanol (Rathburn Chemicals Ltd, Walkerburn, UK) to give a concentration of 1.00 mg/mL. Ten calibration standards (1,000, 500, 250, 100, 50, 25, 10, 1, 0.5 and 0.1 μg/L) were prepared by appropriate dilution of the stock methanolic solutions in blank donor urine for all drugs measured. Internal quality controls (QCs) at three levels (5, 35 and 350 μg/L) were independently prepared in the same way.

Deuterated amlodipine (amlodipine-d_4), bisoprolol (bisoprolol-d_5), doxazosin (doxazosin-d_8), hydrochlorothiazide (hydrochlorothiazide 13C,d2), oxazepam (oxazepam-d_5), morphine (morphine-d_3) ramipril (ramipril-d_5) and (±)-11-nor-9-carboxy-Δ^9-THC (THC-COOH-d_3) were used as internal standards (Cerilliant and LGC) at a working concentration of 100 μg/L in HPLC-grade water (Rathburn Chemicals) containing 0.1% formic acid and 1 mM ammonium formate (Sigma).

Sample preparation

Standards or samples (50 μL) were manually pipetted into a 1.1-mL screw-topped conical glass vial (Kinesis Solutions, Cambridgeshire, UK). To this, 150 μL of working internal standard was added. Samples were vortex-mixed for 10 s and transferred to the autosampler for analysis. About 20 μL of sample was injected and analyzed by LC–MS-MS.

Creatinine in urine was measured using the kinetic alkaline picrate method on the Abbott Architect c-8000 analyzer (Abbott Diagnostics, Abbott Park, IL, USA).

LC–MS-MS

The instrumentation consisted of a Shimadzu high-performance liquid chromatograph (Shimadzu, Milton Keynes, UK) and an API 4000 tandem mass spectrometer (AB Sciex, Warrington, UK) using an electrospray ionization ion source. The column used was a Hypersil Gold column (1.9 μm, 100 mm × 2.1 mm; Thermo Scientific, Hemel Hempstead, UK) with a Gemini C-18 Guard Column (4 × 3 mm; Phenomenex, Cheshire, UK) both maintained at 60°C. The mobile phases utilized were (A) HPLC-grade water containing 0.1% formic acid and 1 mM ammonium formate (Sigma) and (B) 90% HPLC-grade acetonitrile (Rathburn Chemicals) containing 0.1% formic acid and 1 mM ammonium formate. Gradient

Table I. Antihypertensive Agents Assayed in the Urine and their Primary Mode of Action

Class of drug	Examples
Calcium channel blockers (CCBs)	Amlodipine, diltiazem, felodipine, verapamil, nifedipine
Angiotensin-converting enzyme (ACE) inhibitors	Lisinopril, perindopril, ramipril, enalapril
Angiotensin receptor blockers (ARB)	Losartan, irbesartan, candesartan
Diuretics	Indapamide, furosemide, bendrofluomethiazide, hydrochlorothiazide
Sympathetic blockers (β- and α-blockers)	Atenolol, labetalol, bisoprolol, doxazosin, metoprolol
Others	Spironolactone (aldosterone receptor antagonist), moxonidine (imidazoline receptor subtype 1 agonist)

elution was employed for the analysis with the proportion of mobile phase B being maintained at 5% for 0.5 min, and then increased to 100% by 3.3 min and held at 100% for 0.7 min. Percentage mobile phase B was then reduced to 5% for 1 min giving a total run time of 5.5 min. The flow rate was 0.5 mL/min. The mass spectrometer parameters are given in Table II. To optimize the analysis of all drugs screened, each sample was run twice, once in positive ionization mode and once in negative ionization mode. Representative chromatographs at a standard concentration of 25 µg/L are shown in Figure 1.

Table II. Mass Spectrometer Settings for all Drugs Screened For

Drug	Precursor ion (*m/z*) (ionization mode)	Product ion (*m/z*)	Collision energy (V)	Declustering potential (V)	Dwell time (ms)	Retention time
Amlodipine	409.0 (+)	170.0 (Quant)	44.0	59.0	20	3.08
		208.0 (Qual)	38.0	56.0	20	
Atenolol	267.0 (+)	56.3 (Quant)	50.3	79.3	20	1.78
		144.9 (Qual)	36.0	82.0	20	
Bendroflumethiazide	421.1 (−)	329.0 (Quant)	−37.0	−123.0	70	3.19
		290.0 (Qual)	−32.0	−123.0	70	
Bisoprolol	326.0 (+)	91.0 (Quant)	25.9	91.0	20	2.72
		74.0 (Qual)	42.5	95.5	20	
Candesartan	441.0 (+)	192 (Quant)	39.7	86.8	20	3.18
		207 (Qual)	37.1	77.9	20	
Carenone (spironolactone)	341.0 (+)	107.0 (Quant)	41.0	128.0	50	3.61
Diltiazem	414.0 (+)	178.2 (Quant)	33.9	86.8	20	2.90
		150.3 (Qual)	59.2	150.3	20	
Doxazosin	452.0 (+)	344.0 (Quant)	41.6	129.0	20	2.78
		247.0 (Qual)	55.6	131.5	20	
Enalapril	377.0 (+)	234.2 (Quant)	27.4	81.6	20	2.77
		160.0 (Qual)	38.4	81.6	20	
Felodipine	384.0 (+)	338.0 (Quant)	15.5	75.0	20	3.98
		324.0 (Qual)	34.4	75.8	20	
Furosemide	329.3 (−)	205.0 (Quant)	−29.3	−65.8	70	2.93
Hydrochlorothiazide	295.9 (−)	205.1 (Quant)	−33.9	−81.7	70	1.86
		125.9 (Qual)	−42.4	−118.4	70	
Indapamide	366.0 (+)	90.9 (Quant)	55.4	92.0	20	3.08
		117.0 (Qual)	58.0	86.0	20	
Irbesartan	429.0 (+)	207.0 (Quant)	34.0	103.0	20	3.32
		180.0 (Qual)	58.0	108.0	20	
Labetalol	329.0 (+)	161.9 (Quant)	35.9	80.4	20	2.61
		91.0 (Qual)	60.4	70.6	20	
Lisinopril	406.0 (+)	84.0 (Quant)	45.5	92.8	50	2.08
Losartan	423.4 (+)	207.2 (Quant)	35.0	75.0	20	3.23
		180.0 (Qual)	55.0	108.2	20	
Metoprolol	268.0 (+)	116.0 (Quant)	25.5	115.0	20	2.43
		191.0 (Qual)	26.6	73.0	20	
Moxonidine	242.0 (+)	56.2 (Quant)	66.6	96.1	20	1.77
		136.1 (Qual)	42.9	57.9	20	
Nifedipine	347.0 (+)	254.2 (Quant)	25.7	71.0	20	3.39
		211.1 (Qual)	26.3	71.0	20	
Perindopril	369.0 (+)	172.3 (Quant)	30.0	75.0	50	2.87
		98.1 (Qual)	49.0	82.0	50	
Ramipril	417.0 (+)	117.2 (Quant)	55.0	78.0	50	3.07
		130.2 (Qual)	43.0	78.0	50	
Verapamil	455.0 (+)	303.4 (Quant)	37.0	116.0	20	3.08
		164.8 (Qual)	34.9	116.0	20	
Amlodipine-d_4	413.0 (+)	170.0	44.0	59.0	20	3.08
Bisoprolol-d_5	331.0 (+)	74.0	42.5	95.5	20	2.72
Doxazosin-d_8	458.6 (+)	351.4	43.2	118.2	20	2.78
Hydrochlorothiazide 13C,d2	299.0 (−)	77.8	−45.6	−117.2	20	1.86
Morphine-d_3	289.0 (+)	153.0	57.2	114.6	20	1.08
Oxazepam-d_5	292.1 (+)	246.2	31.6	108.0	20	3.14
Ramipril-d_5	421.6 (+)	121.4	56.2	95.6	20	3.07
THC-COOH-d_3	346.1 (−)	302.0	−28.7	−148.7	30	4.17

Generic mass spectrometer settings were as follows: entrance potential 10 V, collision cell exit potential 10 V, collision gas = 9 V, curtain gas = 40 V, ion source gas 1 = 40 V, ion source gas 2 = 55 V, ion spray voltage = 5,500 V, temperature = 550°C.

V, volts; Quant, quantifier transition; Qual, qualifier transition.

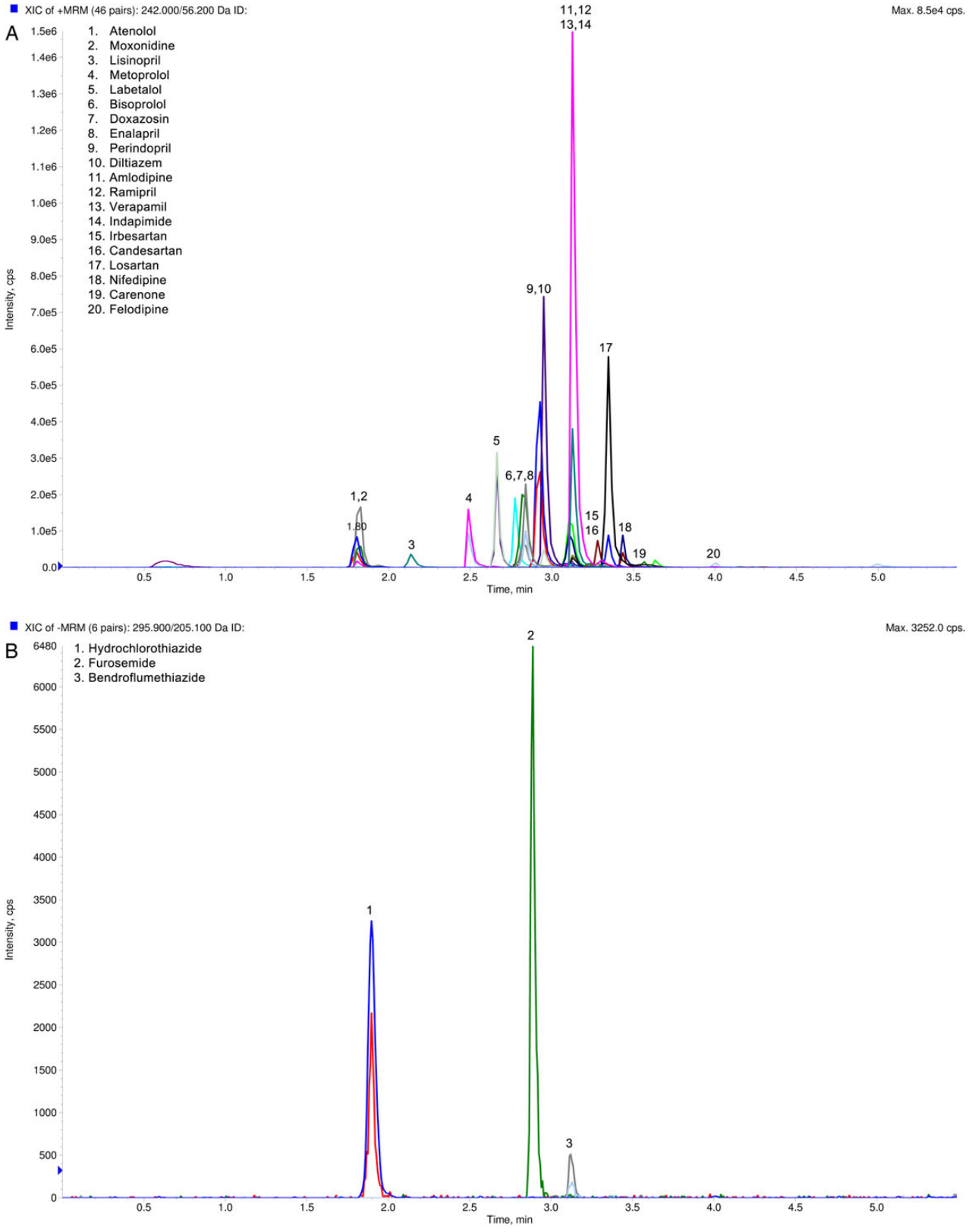

Figure 1. Representative chromatographs from a standard containing all drugs at a concentration of 25 µg/L run in positive (A) and negative (B) ionization modes. This figure is available in black and white in print and in color at *JAT* online.

Method validation

Selectivity

Drug-free urine samples from six healthy volunteers and a drug-free urine sample fortified with a mixture of commonly prescribed medications (Box 1) were processed and analyzed to test the selectivity of the method. The lack of peaks at retention times expected for analytes indicated acceptable selectivity and an absence of interfering substances in the urine.

Box 1 Commonly Prescribed and Over-The-Counter Medications Fortified into Blank Donor Urine to Test Specificity of Method.

Amitriptyline, brompheniramine, caffeine, carbamazepine, citalopram. clomipramine, codeine, cyclizine, desmethylcitalopram, desmethyltramadol, diazepam, dihydrocodeine, diphenhydramine, dothiepin, fluoxetine, gabapentin, glibenclamide, gliclazide, glipizide, ibuprofen, lamotrigine, levetiracetam, mirtazepine, morphine, naproxen, nordiazepam, nortriptyline, olanzapine, oxycodone, paracetamol, paroxetine, pethidine, phenobarbitone, pregabalin, promethazine, quetiapine, quinine, salicylate, sildenafil, theophylline, tolbutamide, trazodone, venlafaxine and zopiclone.

Linearity, lower limit of detection and lower limit of quantitation

Calibration standards at nine concentration levels were freshly prepared as outlined above. The linearity of the assay was calculated by a least-squares linear regression analysis of the peak area ratios of analyte to internal standard versus nominal analyte concentration.

The regression parameters of slope, intercept and correlation coefficient were calculated by the weighting factor, $1/x^2$. Linearity was assessed separately in triplicate using the coefficient of determination (r^2) and by determining the error between nominal and measured concentration for each point on the calibration curve. An acceptable r^2 value was deemed to be >0.95.

The area of blank urine sample was no more than 20% of lower limit of quantitation (LLOQ), where LLOQ was defined as the lowest detectable analyte concentration for which the values of precision (relative standard deviation, RSD) and accuracy (relative error, RE) were ≤20% and the signal-to-noise (S/N) ratio was ≥30. The error was evaluated by precision (RSD) and accuracy (RE) values, which were no greater than 20% for the other seven concentrations in the calibration curve. RE was calculated using RE (%) = [(measured concentration − nominal concentration)/nominal concentration] × 100. Lower limit of detection (LLOD) was defined as the analyte concentration with an S/N ratio of >10.

Precision, accuracy, recovery and matrix effect

QC urine samples were prepared as outlined above and were analyzed on the same day, and over three consecutive days, to evaluate precision and accuracy. Standard curves for each batch were prepared and analyzed on the same day to calculate the concentration of each QC sample. RSD and RE were calculated to estimate precision and accuracy. Recoveries were estimated at a QC concentration relevant for the linear range for each drug measured. The total recovery was evaluated by comparison of the concentration of drug obtained when either a standard amount of a solution containing known amount of drug or a water blank was added to an analytical standard, in a set of five separate experiments. Formula used was: Recovery (%) = [(measured concentration of analyte in fortified sample − measured concentration of analyte in diluted sample)/concentration of spike] × 100.

The matrix effect was assessed in six separate experiments at a relevant QC concentration by comparing the concentration of drug measured when the material was made either in HPLC-grade water or drug-free donor urine. Mean percentage difference between the two should ideally be <10% and statistically insignificant when the replicates are compared using a paired *t*-test. Statistical analysis was performed using Analyse-it.

Stability

The stability of the analyte in urinary samples was analyzed at 1–3 QC levels in triplicate after 24 h at room temperature (25°C), after 7 days at 4°C, after three complete freeze–thaw cycles from −20 to 25°C and after long-term storage at −20°C (28 days). A sample was considered to be stable in the biological matrix when the calculated concentrations were 80–120% of those of the freshly prepared samples.

Clinical effectiveness of the method

The method was applied to the analysis of random urine samples collected from a cohort of patients attending the hypertension clinic at Birmingham Heartlands Hospital. The patients volunteered to give a urine sample and provided a list of all prescribed medication taken in the 24 h prior to clinic attendance. The medication prescribed, dose taken and time of administration (where available) were recorded on the request form. Urine samples were transported to the laboratory within 6 h of collection and stored at −20°C until analysis. For drugs where two transitions are defined (quantifier and qualifier, Table II), both transitions had to be present at the correct retention time (within 0.2 min when compared with an analytical standard and the internal standard used) in order for the specimen to be positive to the drug in question. For those drugs where only one transition could be defined (furosemide, lisinopril and carenone), the presence of this transition at the correct retention time constituted a positive result. Transition ratios were not used for identification. In cases where concentrations of drug were above the linear range of the method, samples were diluted in blank donor urine to a concentration within the linear range, as suggested by the estimated concentration obtained. Samples were then reanalyzed. Drugs identified, and the concentration of these drugs in urine, are summarized in Tables VII and VIII.

Results

The 23 commonly used antihypertensive drugs belonging to various classes (Table I) were separated based on a retention time and MS–MS fragmentation. Although total chromatographic separation was not achieved in positive mode, all compounds could be separated based on drug-specific MS–MS fragmentation with no interference observed between drugs. Owing to the characteristics of the drugs under investigation, and to ensure the optimal sensitivity of the method, samples were run twice, once in positive mode and once in negative mode (Figure 1). Each run was 5.5 min in length, giving a total run time of 11 min per sample.

Selectivity

Typical chromatograms for all drugs screened for are depicted in Figure 1. No interfering signals were observed from the blank donor urine samples tested, or from the urine fortified with drugs listed in Box 1. Retention times were within 0.2 min in each case when compared with an analytical standard and the relevant internal standard.

Table III. Linearity and Recovery Results for All Drugs Screened For

Drug	LLOD	LLOQ	ULOQ	r^2	Recovery (%)
Amlodipine	1	25	250	0.985	64.4
Atenolol	1	10	100	0.980	88.1
BFZ	1	10	1,000	0.990	90.9
Bisoprolol	1	25	1,000	0.978	105
Candesartan	1	10	1,000	0.987	97.0
Carenone	1	10	500	0.972	100.9
Diltiazem	1	25	1,000	0.985	112.5
Doxazosin	1	10	250	0.981	107
Enalapril	0.5	1	500	0.988	85.1
Furosemide	1	10	1,000	0.988	94.3
Hydrochlorothiazide	0.5	10	1,000	0.985	100.1
Indapamide	1	10	500	0.982	90.7
Irbesartan	0.1	1	250	0.981	88.3
Labetalol	0.1	1	250	0.979	83.3
Lisinopril	0.5	1	1,000	0.995	89.2
Losartan	1	1	500	0.994	86.8
Metoprolol	0.5	25	500	0.976	103.5
Moxonidine	0.5	10	250	0.978	84.2
Nifidepine	0.5	10	500	0.995	93.2
Perindopril	0.1	0.5	250	0.998	87.2
Ramipril	0.1	1	1,000	0.988	95.3
Verapamil	1	25	1,000	0.985	119.6

Concentrations of drug are given in μg/L. Felodipine could be reliably detected with an LLOD of 10 μg/L (S/N of 14.9); however, accurate quantitation of drug could not be performed due to unacceptable RSD and RE when assessed for linearity.

BFZ, bendroflumethiazide.

Linearity, LLOD and LLOQ

Linearity, LLOQ and LLOD for all drugs are presented in Table III. Felodipine could be reliably detected with an LLOD of 10 μg/L; however, accurate quantitation of the drug could not be performed due to unacceptable RSD and RE when assessed for linearity.

Precision, accuracy, recovery and matrix effect

Precision and accuracy are summarized in Table IV. Intra- and interday precision for all drugs ranged from 3 to 11.8% at all QC concentrations, with REs for drugs ranging from 0.3 to 19.4%. The precision and accuracy of the assay were thus found to be satisfactory. Recovery experiments are summarized in Table III. Total recoveries for all drugs were within 20% of the fortified value with the exception of amlodipine, which showed a low recovery of drug (64.4%; Table III). The majority of analytes tested displayed no matrix effects shown by statistically insignificant differences of <10% when drug standards were prepared in either water or urine (Table V). Hydrochlorothiazide showed a small (4.8%) significant difference between water and urine, whereas doxazosin, verapamil and candesartan all showed significant increases ($P < 0.05$) in analyte concentration (10–20%) when drug was prepared in urine (Table V). Internal standards used for quantitation were picked to give the lowest amount of matrix effect for the drug being measured.

Stability

Stability data for all drugs are reported in Table VI. All drugs were stable in all conditions tested, with the exception of nifedipine and bendroflumethiazide. Nifedipine levels were 49% lower than the nominal value of 35 μg/L when stored at 25°C for 24 h, whereas

Table IV. Accuracy and Precision Results for All Drugs Screened For

Drug	Intraday ($n = 5$)				Interday ($n = 3$)			
	Nominal concentration (μg/L)	Measured concentration (μg/L) (mean ± SD)	RSD (%)	RE (%)	Nominal concentration (μg/L)	Measured concentration (μg/L) (mean ± SD)	RSD (%)	RE (%)
Amlodipine	35.0	28.4 ± 6.4	6.4	18.1	35.0	30.7 ± 0.3	0.3	−12.3
Atenolol	35.0	34.4 ± 3.7	10.6	−1.8	35.0	35.8 ± 0.8	2.3	2.2
BFZ	35.0	41.3 ± 4.4	10.8	18.0	35.0	35.8 ± 1.0	2.7	2.3
Bisoprolol	35.0	32.8 ± 1.9	5.9	−6.2	35.0	37.4 ± 3.3	8.8	6.8
Candesartan	35.0	34.8 ± 2.8	8.0	0.5	35.0	35.5 ± 0.9	2.5	1.5
Carenone	35.0	39.9 ± 3.0	7.8	14.0	35.0	34.8 ± 1.2	3.4	−0.5
Diltiazem	35.0	29.1 ± 1.4	4.7	−16.7	35.0	32.1 ± 2.2	7.0	−8.4
Doxazosin	35.0	32.0 ± 3.3	10.2	−8.4	35.0	35.9 ± 3.4	9.6	2.5
Enalapril	35.0	37.2 ± 3.3	9.0	6.3	35.0	34.5 ± 2.8	8.0	−1.3
Furosemide	35.0	32.7 ± 1.6	4.7	−6.5	35.0	34.3 ± 0.8	2.2	−2.1
Hydrochlorothiazide	35.0	39.5 ± 3.9	9.8	12.8	35.0	33.9 ± 1.7	4.9	−3.1
Indapamide	35.0	37.8 ± 2.2	5.9	7.9	35.0	34.5 ± 1.3	3.8	−1.5
Irbesartan	35.0	33.3 ± 3.6	10.7	−4.8	35.0	33.8 ± 2.1	6.2	−3.3
Labetalol	35.0	41.8 ± 1.3	3.0	19.4	35.0	36.3 ± 3.3	9.0	3.6
Lisinopril	5.0	5.1 ± 0.5	9.3	1.7	5.0	5.4 ± 0.04	0.8	7.0
Losartan	35.0	32.9 ± 2.8	8.6	−6.0	35.0	31.6 ± 1.4	4.6	−9.8
Metoprolol	35.0	36.4 ± 1.4	3.8	4.0	35.0	37.7 ± 0.8	2.2	7.8
Moxonidine	35.0	37.0 ± 2.6	7.0	5.6	35.0	38.4 ± 0.2	0.5	9.8
Nifidepine	35.0	38.4 ± 3.5	9.1	9.8	35.0	34.4 ± 2.2	6.5	−1.6
Perindopril	5.0	5.5 ± 0.2	4.2	10.8	5.0	5.3 ± 0.0	0.2	5.9
Ramipril	5.0	5.4 ± 0.6	11.8	9.1	5.0	5.2 ± 0.1	2.5	4.4
Verapamil	35.0	31.5 ± 3.4	10.9	−9.9	35.0	37.0 ± 1.1	2.9	5.7

All values correct to 1 decimal place.

BFZ, bendroflumethiazide.

Table V. Effect of Matrix on Measurement of Antihypertensive Medications

Drug	Water (μg/L)	Urine (μg/L)	% Difference	*P*-value	Internal standard
Amlodipine	29.7 (0.5)	29.4 (2.8)	−1.0	0.67	Amlodipine-d_4
Atenolol	34.4 (3.7)	35.8 (4.4)	+4.1	0.65	Morphine-d_3
BFZ	41.3 (4.4)	40.1 (4.4)	−2.9	0.36	THC-COOH-d_3
Bisoprolol	48.2 (5.8)	49.6 (3.3)	+2.8	0.26	Bisoprolol-d_5
Candesartan	34.8 (2.8)	38.4 (1.2)	+10.2	0.05*	Oxazepam-d_5
Carenone	39.9 (3.1)	39.5 (1.5)	−1	0.72	Oxazepam-d_5
Diltiazem	42.3 (3.2)	41.1 (3.6)	−2.9	0.18	Amlodipine-d_4
Doxazosin	32.9 (2.1)	37.5 (3.0)	+14	0.02*	Doxazosin-d_8
Enalapril	51.2 (2.0)	51.6 (2.3)	+0.4	0.42	Ramipril-d_5
Furosemide	298.0 (26.8)	306.6 (44.4)	+2.9	0.77	THC-COOH-d_3
Hydrochlorothiazide	49.8 (2.0)	47.4 (1.5)	−4.8	0.03*	Hydrochlorothiazide 13C,d2
Indapamide	49.1 (3.0)	48.4 (1.8)	−1.3	0.90	Ramipril-d_5
Irbesartan	56.1 (2.8)	56.3 (2.2)	+0.4	0.67	Ramipril-d_5
Labetalol	48.2 (3.2)	49.9 (1.2)	+3.7	0.24	Bisoprolol-d_5
Lisinopril	42.4 (1.7)	42.3 (1.5)	−0.2	0.59	Ramipril-d_5
Losartan	41.2 (1.4)	42.0 (2.3)	+1.9	0.19	Ramipril-d_5
Metoprolol	55.3 (2.5)	54.7 (2.2)	−1.0	0.27	Bisoprolol-d_5
Moxonidine	37.0 (2.6)	38.1 (3.0)	+3.1	0.63	Morphine-d_3
Nifidepine	51.0 (1.9)	49.9 (0.5)	−2.2	0.81	Ramipril-d_5
Perindopril	73.3 (2.0)	75.1 (2.6)	+2.5	0.24	Ramipril-d_5
Ramipril	51.2 (2.1)	51.3 (1.3)	+0.2	0.30	Ramipril-d_5
Verapamil	31.0 (2.6)	34.8 (1.0)	+12.1	0.02*	Amlodipine-d_4

Drugs were measured in either HPLC-grade water or blank donor urine. The mean ($n = 6$) amount of drug measured in each matrix is shown with standard deviation given in parentheses. No matrix effect was defined as a mean percentage difference between the two of <10% and statistically insignificant when the replicates are compared using a paired *t*-test at 95% confidence. Values marked with asterisks are classed as significant. Hydrochlorothiazide showed a small (4.8%) significant difference between water and urine, whereas doxazosin, verapamil and candesartan all showed significant increases ($P < 0.05$) in analyte concentration when drug was prepared in urine. Internal standards used for quantitation were picked to give the lowest amount of matrix effect for the drug being measured.

Table VI. Stability Results for All Drugs Screened For

Drug	Nominal concentration (μg/L)	24 h at 25°C	7 days at 4°C	7 days at −20°C	Freeze–thaw (three cycles of −20 to 25°C)	28 days at −20°C
Amlodipine	35.0	30.9 ± 2.2	36.3 ± 3.2	32.6 ± 3.5	36.7 ± 1.2	38.9 ± 2.6
Atenolol	35.0	32.8 ± 3.2	36.1 ± 0.8	36.3 ± 3.1	35.7 ± 1.2	38.8 ± 1.9
BFZ	35.0	9.6 ± 1.7	21.1 ± 2.5	29.0 ± 1.3	29.4 ± 2.5	34.6 ± 2.8
Bisoprolol	35.0	37.3 ± 1.8	38.5 ± 1.9	41.5 ± 2.4	36.8 ± 4.2	35.1 ± 3.6
Candesartan	35.0	37.0 ± 3.8	35.8 ± 3.9	37.0 ± 2.8	39.2 ± 3.2	35.6 ± 1.8
Carenone	35.0	29.4 ± 1.9	33.6 ± 4.6	35.9 ± 0.7	40.3 ± 2.3	37.2 ± 2.0
Diltiazem	35.0	36.5 ± 3.6	32.2 ± 3.5	36.4 ± 5.6	39.7 ± 3.5	34.4 ± 3.7
Doxazosin	35.0	29.7 ± 1.1	39.5 ± 2.7	40.2 ± 3.4	38.4 ± 6.3	38.7 ± 3.8
Enalapril	35.0	36.6 ± 1.4	36.4 ± 2.4	40.5 ± 1.9	40.2 ± 2.0	39.4 ± 3.3
Furosemide	35.0	28.7 ± 2.7	33.2 ± 3.3	29.8 ± 3.7	30.3 ± 1.1	36.4 ± 2.4
Hydrochlorothiazide	35.0	30.4 ± 1.5	30.6 ± 2.9	31.7 ± 1.0	35.6 ± 2.6	33.2 ± 1.5
Indapamide	35.0	38.6 ± 2.5	37.4 ± 3.0	40.5 ± 0.6	39.4 ± 2.5	37.3 ± 3.5
Irbesartan	35.0	29.6 ± 3.0	32.6 ± 3.5	30.7 ± 1.3	37.3 ± 0.2	37.0 ± 1.5
Labetalol	35.0	37.0 ± 1.0	38.1 ± 3.6	37.7 ± 3.0	34.5 ± 2.4	34.2 ± 4.0
Lisinopril	5.0	5.6 ± 0.1	5.1 ± 0.2	4.9 ± 0.6	5.3 ± 0.8	4.8 ± 0.2
Losartan	35.0	30.9 ± 1.7	37.8 ± 0.3	36.8 ± 2.7	39.6 ± 4.0	38.2 ± 2.8
Metoprolol	35.0	32.9 ± 1.8	32.0 ± 1.9	36.0 ± 2.1	33.2 ± 3.6	35.1 ± 2.4
Moxonidine	35.0	38.3 ± 1.5	37.6 ± 3.1	33.6 ± 3.7	35.3 ± 3.3	37.1 ± 1.9
Nifidepine	35.0	17.8 ± 1.8	37.0 ± 5.6	37.4 ± 2.8	39.2 ± 2.9	36.1 ± 3.8
Perindopril	5.0	5.2 ± 0.2	5.3 ± 0.1	5.4 ± 0.2	5.3 ± 0.8	4.9 ± 0.1
Ramipril	5.0	4.9 ± 0.2	5.0 ± 0.3	4.7 ± 0.4	5.2 ± 0.1	5.1 ± 0.4
Verapamil	35.0	36.9 ± 1.1	36.0 ± 1.7	37.6 ± 2.2	39.2 ± 2.0	34.9 ± 1.1

All values are mean concentration of drug in μg/L ± SD ($n = 3$).
BFZ, bendroflumethiazide.

bendroflumethiazide declined from 35 μg/L by 73% when stored at 25°C for 24 h and by 40% when stored at 4°C for 7 days.

Clinical application

To validate the application of the assay, urine samples from a cohort of 49 individuals were screened for the presence of antihypertensive medication. All were attending the hypertension clinic at Birmingham Heartlands Hospital and were taking at least one antihypertensive agent (median 2, range 1–6) present in the LC–MS-MS screening profile. Patients volunteered information on which of their medications had been taken prior to the appointment, normally the night before, but adherence could not be confirmed prior to sampling. Drugs identified, and the concentration of these drugs in urine, are given in Tables VII and VIII. For the majority of samples screened (88%), analysis by LC–MS-MS confirmed patient adherence to the prescribed medication (Table VI). An example of a patient adherent to all medications prescribed is shown in Figure 2. In three patients (representing 8% of samples screened) who were all prescribed one or more of the following: lisinopril, felodipine, furosemide, diltiazem, indapamide, doxazosin, amlodipine and hydrochlorothiazide, no drugs were detected. These drugs were easily detected in individuals considered to be compliant (Table VIII). In the remaining five patients, ramipril was not detected (4% of samples screened)

Discussion

Poor adherence to prescribed medication is thought to be the most important cause of treatment-resistant hypertension (14, 24), with an estimated 50% of long-term medications not taken correctly (16, 25). Good perceived drug tolerability, patient education, reduction in tablet numbers and regime complexity, home BP recording and behavioral counseling have all been recommended as interventions to improve medication adherence (16). More recently, single-pill fixed-dose combination treatment has been shown to improve adherence to antihypertensive medication (26). However, an objective test for drug adherence would be of great use in order to identify individuals who are truly nonadherent and will benefit from such interventions.

This has led to the development of an LC–MS-MS method to screen for the presence of 23 commonly used antihypertensive drugs in urine to monitor adherence to prescribed medication. The method is rapid with minimal sample preparation and is sufficiently sensitive to support clinical decision-making regarding adherence to drug treatment protocols, with the majority of drugs having an LLOD of 0.1–1 μg/L. Linearity, precision, accuracy and recovery for drugs assayed were all within stated tolerances with the exception of felodipine, where RSD and RE were unacceptable when assessed for linearity. However, felodipine was detected in all four cases in which patients were prescribed the drug (an example is shown in Figure 2). The majority of analytes were found to be stable in urine in all situations assessed, with time points and situations used representative of storage and transport conditions which would be encountered in routine clinical practice. The exceptions were nifedipine (unstable when stored at 25°C for 1 day) and bendroflumethiazide (unstable when stored at 25°C for 1 day and when stored at 4°C for 7 days). Both drugs were, however, stable when stored at −20°C for 28 days, indicating that as long as samples are promptly frozen

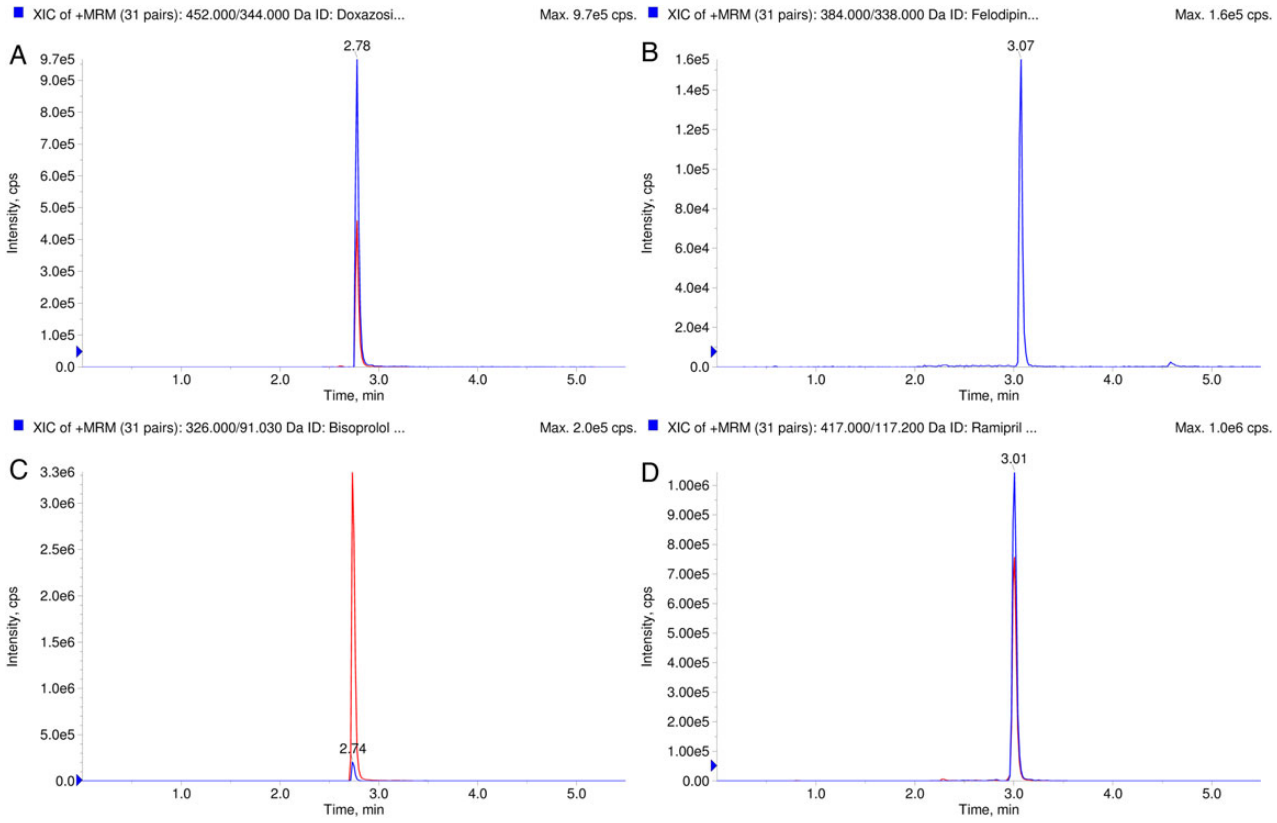

Figure 2. Example extracted ion chromatograms (XIC) from a patient adherent to four prescribed medications: doxazosin (A), felodipine (B), bisoprolol (C) and ramipril (D). For each drug, the two lines represent the quantifier and qualifier transitions. Measured concentrations of drugs in this sample were 12.5, 721.4 and 12.6 μg/mmol creatinine for doxazosin, bisoprolol and ramipril, respectively. Felodipine was not quantified. This figure is available in black and white in print and in color at *JAT* online.

Table VII. Clinical Validation of Assay

Patients screened	49
Drugs screened for based on prescribing information	133
Drugs detected	117 (88)
Drugs not detected	16 (12)
Drugs missed due to potential nonadherence[a]	11 (8)
True false-negatives[b]	5 (4)
True detected	128 (96)

Percentage of drugs not detected is shown in parentheses.

[a]Potential nonadherence was defined as an absence of all drugs prescribed in urine.

[b]This represents five cases where ramipril was not detected in patients who may have taken the drug.

prior to analysis, nifidepine and bendroflumethiazide may be reliably detected by this method.

To validate its clinical effectiveness, the assay has been applied to the analysis of urine collected from 49 patients who had admitted to taking their prescribed antihypertensive agents as directed. Creatinine was also measured in these samples in order to normalize the amount of drug detected to a standard amount of creatinine in urine. This will attempt to correct for any variation in the concentration of urine, which may occur due to differences in the hydration status of the patients under investigation. As provided in Table VII, the LC–MS-MS method was able to detect the vast majority of medications prescribed with only 16 examples (12% of drugs) where prescribed medication was not detected. This included three individuals who were noncompliant to all drugs prescribed and five patients in whom ramipril could not be detected, four of whom were prescribed other medications which was detected by the assay and one to whom ramipril was the sole agent prescribed. Since adherence to ramipril could not be confirmed, it is unclear as to whether these five cases are true nonadherent individuals or whether levels of urinary ramipril in compliant individuals are <0.1 µg/L. Previously reported pharmacological data have shown that a single oral labeled ramipril dose is eliminated primarily in the urine (56%) over a 22-day period with the urinary excretion of unchanged drug being ~2% of a dose, ramiprilat >10% and two diketopiperazine rearrangement products also detectable (27). However, since ramipril was detected in 11 other patients prescribed this drug (as shown in Table VII and Figure 2) at concentrations well above the limit of detection of the assay, nonadherence appears to be the more logical conclusion. Another explanation for the five patients in whom ramipril could not be detected could potentially be the stability of ramipril in urine with *in vitro* breakdown of the drug in urine creating a false-negative result. While hydrolysis of ramipril may be seen as an alternative explanation for this finding, it is unlikely since it has previous been reported that ramipril is stable in aqueous solution at neutral or acid pH (as was the case with these specimens) (27) and reflected by our data, illustrating the stability of ramipril over a variety of conditions (Table V). Taken together, these data endorse the reliability, sensitivity and clinical effectiveness of the assay for the detection of all 23 commonly used antihypertensive medications in urine and denote adherence in this patient cohort of ~94%.

Future improvements to the assay could include the addition of more medications to increase the breadth of drugs screened for (e.g., amiloride) and also the addition of drug metabolites, such as ramiprilat, which could increase the window of detection for drugs and potentially identify those patients who are truly adherent (detection of parent drug and metabolite) compared with those who have taken medication recently in order to pass an adherence test (only parent drug detected). However, it could be seen as an advantage by only targeting parent compounds as positive results may reflect active treatment. The presence of metabolites could therefore impact on interpretation as the patient could potentially not be adherent, but markers of historic drug use would still be

Table VIII. Concentrations of Drug Identified in Urine from the Cohort of Patients Used in This Study

Drug	*n*	µg/L				µg drug/mmol creatinine			
		Median	Maximum	Minimum	IQR	Median	Maximum	Minimum	IQR
Amlodipine	12	445.2	1,110.0	268.0	316.0	88.3	243.7	19.1	105.9
Atenolol	3	2,490.0	3,450.0	1,360.0	1,045.0	2,300.0	4,088.7	529.2	1,779.7
BFZ	4	391.0	1,700.0	94.0	491.5	107.5	291.0	23.3	86.7
Bisoprolol	8	381.0	1,160.0	252.0	144.0	113.4	721.4	33.5	125.6
Candesartan	1	184.0	–	–	–	86.6	–	–	–
Carenone	4	15.3	76.1	18.9	21.6	10.52	23.2	3.4	5.4
Diltiazem	5	531.0	2,070.0	149.0	609.0	108.7	405.9	45.9	127.4
Doxazosin	10	26.2	891.0	10.1	118.4	6.7	174.7	1.9	9.4
Furosemide	12	1,540.0	5,850.0	16.2	4,081.0	307.14	4,267.0	3.6	1,203.2
Indapamide	6	42.0	172.0	11.6	100.6	14.3	36.9	6.0	16.4
Irbesartan	2	38.2	68.4	8.1	30.2	12.7	22.8	2.7	10.1
Labetalol	1	1.4	–	–	–	0.19	–	–	–
Lisinopril	2	3,375.3	6,700.0	50.6	3,324.7	408.3	798.6	18.0	
Losartan	5	108.0	2,320.0	9.6	88.5	38.4	3,809.5	0.4	41.8
Moxonidine	2	61.05	92.3	29.8	31.3	38.8	48.9	28.7	10.1
Perindopril	6	3.8	92.3	0.5	6.3	1.4	5.4	0.3	0.7
Ramipril	11	5.5	178.0	1.0	3.3	1.4	12.6	0.1	5.4
Verapamil	1	537.0	–	–	–	274.0	–	–	–

No patients in our cohort were identified as taking enalapril, metoprolol or nifedipine. One patient in the cohort was prescribed hydrochlorothiazide; however, this drug, together with amlodipine and doxazosin, was not detected in the sample collected indicating nonadherence in this case. Felodipine was not able to be quantified due to reasons outlined previously.

IQR, interquartile range; BFZ, bendroflumethiazide.

detected. While initially intended and currently employed primarily as a screening technique to determine adherence to prescribed medication, the ability to quantitate the drugs detected offers many advantages for on-going monitoring and management of individuals with resistant hypertension. By applying the method described above to the strict monitoring of a group of patients where the dose, time administered and time of sample collection are all known, it may be possible to develop cutoff concentrations for each drug in urine to determine if a patient is adherent or not, similar to other semi-quantitative urine screening assays (e.g., drugs of abuse screening). In addition, as the technique becomes more widely employed, it may become feasible to individualize patient monitoring. By maintaining a database of patient-specific drug concentrations, it may be possible to detect any individual differences in adherence, the impact of other drugs if dosage regimens are altered, or to explain therapeutic failures due to other physiological variables affecting drug absorption or elimination.

Despite the evidence that poor adherence to prescribed medications is the most important cause of resistant hypertension (14, 24), the method outlined in this paper is only the second published LC–MS-MS technique for the detection of a range of antihypertensive drugs in urine. The first, a clinical paper published last year, used LC–MS-MS to screen urine samples from a cohort of 208 hypertensive patients and showed that nonadherence to BP lowering therapy is common, particularly in patients with suboptimal BP control and those referred for renal denervation (28). However, some key analytical parameters, notably the MRM transitions employed for detection, were omitted from the publication preventing its development and use elsewhere. In addition, the method employed lengthy sample preparation and analysis procedures (dilution and solvent extraction incorporating a hydrolysis step), and subsequent analysis in both positive and negative ionization modes with a total analysis time of 48 min per specimen (28). The total sample analysis time, coupled with the large amount of sample required (5 mL), may impact on the suitability of this approach for use in a routine clinical laboratory. In contrast, the method described here is highly sensitive while only using a very small volume of sample (50 μL) with a rapid and simple sample preparation (~30 s per sample) and short analytical run time (11 min). This robust procedure could therefore be easily implemented into a number of clinical laboratories in order to facilitate improvements in the investigation of resistant hypertension on a large scale, or in a real-time setting.

On-going work includes the further application of the assay in clinical practice including evaluating its benefit on the current patient pathway for the management of resistant hypertension. Particular aspects of importance include whether the use of the assay will result in improved patient adherence, the potential cost-saving due to a reduction in the total number of drugs prescribed and an overall improvement in the number of patients successfully treated. Potentially, some immediate benefits can be foreseen for both patient safety and outcome and an associated reduction in the burden on healthcare services; however, data are not available for these parameters yet. Further research is planned comparing urine antihypertensive drug assay with DOT, and assessing the effectiveness of the assay in tailoring interventions to reduce nonadherence and improve BP control. We believe that the development of clinical interventions based on results from the urine antihypertensive drug assay will reduce cardiovascular events and mortality in those with apparent resistance to antihypertensive treatment. This, along with the reduction in medication costs and waste, can potentially result in significant savings to the National Health Service.

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