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A Dilute-and-Shoot LC–MS/MS Method for Screening of 43 Cardiovascular Drugs in Human Urine

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Abstract : A simple, specific, and economical LC–MS/MS method was investigated for the screening of 43 prescribed antihypertensive and related drugs in human urine. The urine samples were simply prepared by diluting and mixing with internal standard before directly introduced to the LC-MS/MS system, which is fast, straightforward, and cost-effective. Fractional factorial, Box-Behnken, and I-optimal design were applied to screen and optimize the mass spectrometric and chromatographic factors. The analysis was carried out on a triple quadrupole mass spectrometer system utilizing multiple reaction monitoring with positive and negative electrospray ionization method. Chromatographic separation was performed on a Thermo Scientific Accucore RP-MS column (50 × 3.0 mm ID., 2.6 μm) using two separate gradient elution programs established with the same mobile phases. Chromatographic separation was performed within 12 min. The optimal method was validated based on FDA guideline. The results indicated that the assay was specific, reproducible, and sensitive with the limit of detection from 0.1 to 50.0 μg/L. The method was linear for all analytes with coefficient of determination ranging from 0.9870 to 0.9981. The intra-assay precision was from 1.44 to 19.87% and the inter-assay precision was between 2.69 and 18.54% with the recovery rate ranges from 84.54 to 119.78% for all drugs measured. All analytes in urine samples were stable for 24 h at 25°C, and for 2 weeks at -60°C. The developed method improves on currently existing methods by including larger number of cardiovascular medications and better sensitivity of 12 analytes.

Keywords : antihypertensive drugs, screening test, dilute-and-shoot LC-MS/MS, experimental design

Introduction

Hypertension and other cardiovascular diseases which have been among the leading cause of death worldwide, are preventable and manageable by medications such as antihypertensive, hypolipidemic, or anticoagulant agents.[1] However, the increasing of non-adherence to antihypertensive and related drugs is a real menace to patient health and drug effectiveness. Several conventional methods have been applied to evaluate medication adherence including questionnaires, pharmacy dispense records, pill counts, or supervised administration.[2] Besides, recently, drug testing in urine, oral fluid, or plasma using liquid chromatography tandem mass spectrometry (LC-MS/MS) has been proven as a valuable means for assessing the adherence of prescribed medications. The developed LC-MS/MS methods for drug adherence monitoring in general and studying cardiovascular medications in particular generally applied sample preparation processes employed solid-phase extraction or liquid-liquid extraction.[3-8] This approach effectively cleans up and concentrates the analytes but significantly depends on the characteristics of the surveyed compounds as well as consumes labor, reagents, and time Nowadays, the enhancement in the sensitiveness of LC–MS/MS systems have allowed samples to be minimally diluted and then directly introduced into the analytical system. This offers a simple and faster sample preparation process (about 30 s) with minimal labor, time and reagent consumption and be able to screen the broader range of analytes in comparison to other mentioned techniques. For instance, “dilute-and-shoot” LC-MS/MS has been proven as an effective trend in doping

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control,[9] analytical toxicology,[10] or urine drug testing of a large number of antipsychotics, opioids, benzodiazepines, and other pain management medications and metabolites.[11-13] As such, a limited number of antihypertensive, lipid-lowering, antihyperglycemic, antithrombotic and other cardiovascular agents were successfully screened in urine applying "dilute-and-shoot" LC-MS/MS method.[14-16] In which, the study of A.J. Lawson covered a largest number of antihypertensive medications but only 23 compounds.[14]

From the above overview, this study developed a "dilute-and-shoot" LC-MS/MS method to detect a larger number of cardiovascular preventive compounds, covering 43 prescribed antihypertensive, lipid-lowering, and antithrombotic agents available worldwide. The design of experiment (DOE) was aslo applied through the method development process to achieve the effectively and reliably optimal LC-MS/MS condition with minimum experiments, time, cost, and labor consumption.[17]

Experimental

Material

43 surveyed cardiac drugs as well as atenolol-d7, and sulfameter (as internal standards (IS)) were provided from Sigma-Aldrich (St. Louis, MO, USA). Other IS including amlodipine-d4, clopidogrel-d4, diltiazem-d3, losartan-d4, telmisartan-d7 were supplied by TLC Pharmaceutical Standard. Formic acid, ammonium formate, HPLC-grade acetonitrile, and methanol were purchased from Daejung (Siheung, South Korea). Distilled water was prepared in the laboratory utilizing an Aqua Max water purification system supplied by Young Lin Instrument Co., Ltd. (Anyang, South Korea).

Instrumentation

The LC-MS/MS system included an Agilent 1200 series (Agilent Technologies) system combined with an API 3200 Q Trap triple-quadrupole mass spectrometer (AB SCIEX) operated with a Turbo V Ion Spray source. Analyst 1.6 software was employed for LC-MS/MS system management and data processing. The separation was performed on a Thermo Scientific Accucore RP-MS column (50 × 3.0 mm ID., 2.6 μm) combined with a C18 guard column (Phenomenex, 4.0 × 3.0 mm ID), both maintained at 50°C. Two separate gradient elution programs established with the same mobile phases: eluent A containing 8mM ammonium formate ($HCOONH_4$) and 0.1% formic acid (HCOOH) in water, and eluent B containing 8mM $HCOONH_4$ and 0.1% HCOOH in acetonitrile (ACN): water (90:10).

Drug calibrators and quality control samples preparation

A 1 mg/mL stock solution in methanol was made for each compound measured and IS, with the exception of 2 mg/mL for nicotinic acid and 5 mg/mL for HCTZ. Therefore, the concentration of nicotinic acid and HCTZ is correspondingly 2 times and 5 times higher than that mentioned the following solutions. Working standard mixtures of 4000 μg/L, 200 μg/L, 10 μg/L and IS working standard mixtures of 4000 μg/L were prepared by serial dissolving the stock solutions in water. All solutions were keeped at -20°C and thawed at room temperature (25°C) before use. Fifteen calibration standards (0.25, 0.5, 1, 2, 5, 10, 20, 30, 50, 100, 200, 400, 600, 800, 1000 μg/L) were prepared by spiking an appropriate volume of the diluted standard solutions into an aliquot containing 250 μL of drug free human urine, and 200 μL of diluted IS solution, followed by dilution with water to attain a total volume of 1000 μL. Quality control (QC) samples correspond with three concentration levels (low, medium, and high) were independently prepared in the same way for all drugs measured. The sample was then vortexed and filtered using 0.45 μm filter before introducing into LC-MS/MS system.

MS analyte parameters

Precursor and product ion transitions of each compound were determined by direct infusion of standard solution with positive and negative electrospray ionization (ESI) source. The multiple reaction monitoring (MRM) transitions and compound tuning parameters are shown in Table 1. According to optimization results, the optimal mode for each compound which created the higher intensity signal was selected (i.e. 39 compounds were detected in a positive ESI method and 4 compounds in a negative ESI method).

In scouting phase, five MS parameters including ion spray voltage, capillary temperature, curtain gas, ion source gas 1, and ion source gas 2 were screened to identify the significant factors by applying fractional factorial design. Peak areas of poorly sensitive compounds (Amlodipine, Atenolol, Captopril, Losartan, Lovastatin, Moxonidine, Nicotinic acid, and Spironolactone) were chosen as responses. Analysis of variance (ANOVA) was utilized to assess the impacts of factors. Selected important factors were then optimized by Box-Behnken design with 15 runs including 3 centre points.

LC parameters

As the analytical column is stable at temperature below 60°C, the influence of the column temperature was studied in a range from 20°C to 50°C with a step of 5°C. Three LC related parameters namely flow rate, ammonium formate concentration, and percentage of eluent B at 0 min were also optimized by I-optimal design with 20 runs. Intensities of poor sensitive compounds were chosen as responses.

Method validation

Selectivity

The selectivity of method was studied by comparing six

drug-free urine samples from six individual sources and drug-free urine samples spiked with a surveyed medications mixture at lower limit of quantification (LLOQ) concentrations. The absence of interfering peaks at retention times of analytes indicated satisfactory selectivity.

Sensitivity

The limit of detection (LOD) was assessed by the analyte concentration with the signal-to-noise (S/N) ratio was > 3. The LLOQ concentration was determined at which the S/N ratio was ≥ 10 as well as the precision (assessed by relative standard deviation, RSD) and variance of accuracy (relative error, RE) were ≤ 20%.

Carryover

The carryover was tested by analyzing the blank samples right away the upper limit of quantification (ULOQ) samples (n = 3). The carryover should ideally be < 20%.

Matrix effect

The matrix factors of the analytes were assessed by comparing the analyte/IS ratio in urine samples and water (solvent) at low, medium, and high concentration in three separate experiments (n = 3). Average percentage difference between the two should preferably be between -20% and 20%.

Linearity

The linearity was tested within the concentration range from LLOQ to ULOQ concentration using a weighting factor of 1/x in the linear regression analysis. Linearity was evaluated basing on the coefficient of determination (R^2) in five replicates. R^2 value of >0.95 indicated acceptable linear.

Table 1. MRM transitions, Compound tuning parameters, and t_R.

Compound	Q1	Q3 (1)	Q3 (2)	ESI	DP (V)	EP (V)	CE1 (V)	CE2 (V)	t_R (min)	IS
Acebutolol	337.2	116.3	56.2	(+)	56	7	27	47	1.85	Ate7
Amlodipine	410.2	239.2	238.2	(+)	21	4	17	17	4.55	Aml4
Aspirin	178.8	93.0	93	(-)	-15	-3.5	-8	-32	4.50	Los4
Atenolol	267.2	145.2	56.2	(+)	26	10	37	41	0.85	Ate7
Atorvastatin	559.4	440.4	250.3	(+)	66	8.5	23	53	5.12	Clo4
Bendroflu-methiazide	420.0	289.1	197.1	(-)	-80	-4.5	-24	-66	5.35	Los4
Betaxolol	308.2	55.1	72.2	(+)	61	6	45	33	4.28	Tel7
Bevantolol	346.2	165.2	150.2	(+)	56	6.5	25	43	4.27	Aml4
Bisoprolol	326.2	116.3	74.1	(+)	51	5.5	23	37	3.98	Tel7
Captopril	218.1	116.1	75.1	(+)	36	7.5	17	27	1.65	Ate7
Carvedilol	407.2	100.0	56.2	(+)	56	7	41	63	4.42	Aml4
Celiprolol	380.2	74.2	251.3	(+)	51	6.5	47	27	3.40	Sul
Clonidine	230.0	74.1	124.0	(+)	56	8.5	101	57	1.00	Ate7
Clopidogrel	322.1	155.2	184.3	(+)	36	4.5	47	33	5.69	Clo4
Diltiazem	415.2	178.2	109.2	(+)	46	5.5	33	85	4.26	Dil3
Doxazosin	452.2	344.4	247.3	(+)	106	10	33	51	4.12	Dil3
Enalapril	377.3	234.3	91.1	(+)	41	6	23	75	4.12	Tel7
Fluvastatin	412.2	354.4	354.5	(+)	66	6.5	19	19	5.12	Clo4
Furosemide	329.0	205.0	284.9	(-)	-45	-4.5	-24	-14	5.02	Los4
Hydrochlorothiazide (HCTZ)	296.6	77.7	270.1	(-)	-50	-5	-48	-14	1.78	Sul
Indapamide	366.1	132.2	91.2	(+)	46	6.5	23	53	4.41	Dil3
Irbesartan	429.2	207.1	205.2	(+)	56	7	31	69	4.83	Tel7
Labetalol	329.2	91.1	162.2	(+)	36	6.5	53	31	3.84	Dil3
Lisinopril	406.2	84.2	91.1	(+)	56	6.5	41	87	0.83	Sul
Losartan	424.2	208.2	207.3	(+)	51	5	27	33	4.69	Los4
Lovastatin	405.3	199.3	173.2	(+)	56	5.5	21	25	5.54	Clo4
Metoprolol	268.2	74.1	56.2	(+)	46	9	33	43	2.13	Sul
Mevastatin	391.3	185.2	159.3	(+)	56	5	25	33	5.40	Tel7
Moxonidine	243.2	207.2	200.1	(+)	66	8	19	27	0.71	Ate7

Table 1. Continued.

Compound	Q1	Q3 (1)	Q3 (2)	ESI	DP (V)	EP (V)	CE1 (V)	CE2 (V)	t_R (min)	IS
Nadolol	310.2	254.3	201.3	(+)	51	6	21	27	1.00	Ate7
Nicotinic acid	124.0	80.1	78.1	(+)	46	10	29	29	0.71	Sul
Olmesartan	559.2	207.2	190.3	(+)	71	6	37	103	4.69	Los4
Perindopril	369.2	172.3	98.1	(+)	46	6	25	49	4.23	Tel7
Pindolol	249.2	116.3	172.2	(+)	46	9	23	21	1.14	Ate7
Pitavastatin	422.2	274.3	290.3	(+)	91	7	61	31	4.83	Dil3
Propranolol	260.2	116.3	56.1	(+)	51	7.5	23	43	4.12	Dil3
Ramipril	417.2	234.3	91.2	(+)	76	5.5	25	91	4.41	Dil3
Rosuvastatin	482.3	258.1	258.3	(+)	81	5	37	37	4.69	Tel7
Spironolactone	341.2	107.2	91.2	(+)	76	7	41	73	4.97	Clo4
Telmisartan	515.2	276.3	261.3	(+)	96	8	65	83	4.83	Tel7
Terazosin	388.1	290.3	247.3	(+)	76	9.5	29	35	1.42	Ate7
Triamterene	254.2	237.3	104.2	(+)	76	12	33	51	1.02	Ate7
Warfarin	309.1	163.1	251.2	(+)	71	6	19	23	4.97	Tel7
Amlodipine-d4	413.2	238.2	298.3	(+)	66	5	19	19	4.41	
Atenolol-d7	274.3	145.2	79.2	(+)	51	6.5	35	33	0.71	
Clopidogrel-d4	326.1	216.2	159.2	(+)	51	6	19	45	5.54	
Diltiazem-d3	418.1	178.1	109.1	(+)	46	6	31	85	4.27	
Losartan-d4	427.2	211.3	210.2	(+)	60	5	43	45	4.55	
	425.1	128	157.2	(-)	-60	-4.5	-40	-36	5.43	
Sulfameter	281.1	65	108.1	(+)	51	5.5	65	33	1.71	
	279	196.1	264.1	(-)	-45	-4.5	-38	-12	3.02	
Telmisartan-d7	522.3	280.4	279.3	(+)	111	12	63	67	4.83	

DP: de-clustering potential, EP: entrance potential, CE: collision energy, t_R: Retention time

Precision and accuracy

The intra-day, inter-day precisions, and accuracy were assessed by analyzing five replicates on same day, and over three different days of four concentrations: LLOQ, low of quantification (LQC), medium of quantification (MQC), and high of quantification (HQC). Standard curves for each batch were prepared and analysed on the same day to determine the concentration of each QC sample. RSD and RE were also calculated to evaluate the precision and accuracy.

Stability

The stability of all compounds in urinary samples was investigated at 3 QC concentrations (LQC, MQC, and HQC) in three replicates. The QC samples were stored under 4 different storage conditions before analyzing: 24 h at room temperature (25°C), 2 weeks at -20°C, three cycles of freezing (-60°C for 12 h) and thawing (room temperature), and autosampler 5°C for 24 h. An analyte was considered to be stable in urine when the calculated concentrations were 85–115% of those of the freshly prepared samples.

Results

Method development

Preliminary experiments were conducted with the following gradient LC condition proposed by Lawson et al.: eluent A including 1mM $HCOONH_4$ and 0.1% HCOOH in water, and eluent B including 1mM $HCOONH_4$ and 0.1% HCOOH in 90% ACN.[14] Some analytes such as captopril, losartan, lovastatin, moxonidine, nicotinic acid, hydrochlorothiazide (HCTZ) or spironolactone showed the poor sensitivity and chromatographic performance, so further experiments were conducted to obtain the more suitable condition.

Optimization of MS parameters

At first, five MS parameters including ion spray voltage, capillary temperature, curtain gas, ion source gas 1, and ion source gas 2 were screened to identify the significant factors by applying fractional factorial design. Since p-value < 0.05, ion spray voltage, capillary temperature, and curtain gas were demonstrated the more importance and selected for optimization step. These MS selected factors

Table 2. The optimization of MS parameters.

		Negative mode	Positive mode
Factors & ranges	Ionspray voltage (V)	-4500 ~ -3500	3000 ~ 5000
	Temperature (°C)	450 ~ 650	450 ~ 650
	Curtain gas (psi)	30 ~ 50	20 ~ 40
Responses		Peak areas of Aspirin, Bendroflumethiazide, Furosemide, HCTZ	Peak areas of Amlodipine, Atenolol, Captopril, Losartan, Lovastatin, Moxonidine, Nicotinic acid, Spironolactone
Total run		15 runs	15 runs
Desirability value		0.954	0.427
Optimal MS values	Ionspray voltage (V)	-4500	4207
	Temperature (°C)	650	637
	Curtain gas (psi)	50	20
	Ion source gas 1 (psi)	60	60
	Ion source gas 2 (psi)	30	70

were optimized by Box-Behnken design with 15 runs including 3 centre points. From the results of Box-Behnken design, optimal MS conditions were revealed. The desirability values were 0.954 and 0.427 for negative and positive mode, respectively (Table 2).

Optimization of LC parameters

The results of column temperature investigation showed that high temperatures faster elution of analytes, improved peak shapes, and obtained the acceptable sensitivity (peak area and peak height). Therefore, the temperature of analytical column was stabled at 50°C in following experiments.

Three other LC related parameters namely flow rate, ammonium formate concentration, and percentage of eluent B at 0 min were also optimized by I-optimal design with 20 runs. Intensities of poor sensitive compounds were chosen as responses. At optimal condition, the desirability values were 0.943 and 0.466 for negative and positive mode, respectively (Table 3).

Overall, there were the significant differences in desirability values between positive and negative mode since the number of responses of positive mode (8) was higher than that of negative one (4). Despite the low desirability, the sensitivity and chromatographic performance of almost surveyed compounds was acceptable and good enough for drug screening method. Therefore, the finally optimal LC-MS/MS was selected following DOE results (Table 2 and 3). The complete chromatograms all analytes were shown in Figure 1.

Method validation

Selectivity and sensitivity

There were no considerable interfering peaks observed at the retention times expected for the analytesf or IS. The extracted ion chromatograms of 43 interested compounds and IS were shown in Supporting Information (Figure S1).

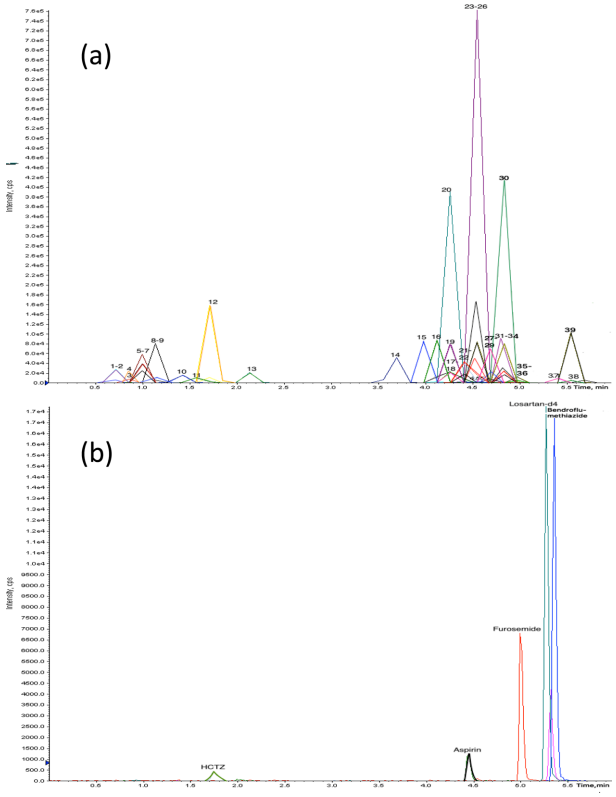

Figure 1. Chromatograms of 40 analytes in a positive ESI mode (a) and 4 analytes in a negative ESI mode (b): 1. Moxonidine, 2. Nicotinic acid, 3. Atenolol, 4. Lisinopril, 5. Clonidine, 6. Nadolol, 7. Triamterene, 8. Enalapril, 9. Pindolol, 10. Terazosin, 11. Captopril, 12. Acebutolol, 13. Metoprolol, 14. Celiprolol, 15. Labetalol, 16. Bisoprolol, 17. Doxazosin, 18. Propranolol, 19. Perindopril, 20. Diltiazem, 21. Bevantolol, 22. Betaxolol, 23. Indapamide, 24. Ramipril, 25. Carvedilol, 26. Amlodipine, 27. Losartan, 28. Olmesartan, 29. Rosuvastatin, 30. Irbesartan, 31. Pitavastatin, 32. Telmisartan, 33. Spironolactone, 34. Warfarin, 35. Atorvastatin, 36. Fluvastatin, 37. Mevastatin, 38. Lovastatin, 39. Clopidogrel.

Table 3. The optimization of LC condition.

		Negative mode	Positive mode
Factors & ranges	Flow rate (mL/min)	0.3 – 0.5	0.3 – 0.5
	Buffer conc. (mM)	2 - 8	2 - 8
	%B at 0 min (%)	10 - 30	10 - 30
Responses		(Similar to MS parameters optimization)	
Total run		20 runs	20 runs
Desirability value		0.943	0.466
Optimal LC condition	Flow rate (mL/min)	0.3	0.37
	Buffer conc. (mM)	8	8
	%B at 0 min (%)	10	15
	Gradient elution - Eluent A: 8 mM $HCOONH_4$ and 0.1% HCOOH in Water - Eluent B: 8 mM $HCOONH_4$ and 0.1% HCOOH in Water - ACN (1:9)	0.0–0.2 min: 10%B 0.2-2.5 min: 10% – 100%B 2.5-6.0 min: 100%B 6.0-7.0 min: 100% – 10%B 7.0-12.0 min: 10%B	0.0–0.2 min: 15%B 0.2-2.5 min: 15% – 100%B 2.5-6.0 min: 100%B 6.0-7.0 min: 100% – 15%B 7.0-12.0 min: 15%B

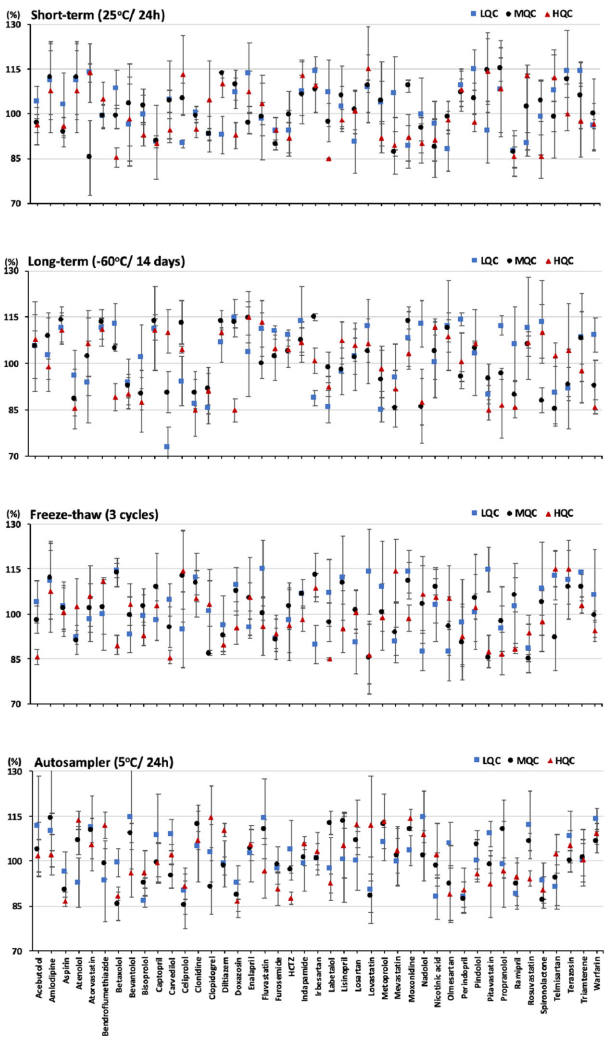

Figure 2. Stability validation.

The LODs were from 0.1 to 50 ppb, and the LLOQs ranged from 0.25 to 100 ppb (Table 4).

Carryover

The carryover of the all surveyed compounds was less than 19.48% of the LLOQ (Table 4).

Matrix effect

Mean percentage difference of the analyte/IS ratio between human urine and water samples was from -19.92% to 18.92% for all but three analytes (bevantolol, carvedilol, nicotinic acid) (Table 4).

Linearity

The coefficient of determination (R^2) of all compounds was more than 0.9870 showing the acceptable linearity of the developed method.

Precision and accuracy

The good precision and accuracy were observed for all compounds (Table 5). The RSD% was not more than 19.87% and 18.54% for intra-assay and inter-assay precision, respectively. The recovery of each compound was in the range from 84.54 to 119.78%.

Stability

The results of stability validation are shown in Figure 2 and Supporting Information (Table S1). Under four storage conditions, the mean of recoveries and RSD satisfied the acceptance criteria (± 15% of the control values) for all analytes but carvedilol (recovery of 73.07% at LQC). No significant degradation was detected, so most analytes were assessed to be stable in urine under all described conditions.

Table 4. QC concentrations, sensitivity, linearity, carry over, matrix effect validation.

Compound	**LOD** (ppb)	**LLOQ** (ppb)	LQC (ppb)	MQC (ppb)	HQC (ppb)	ULOQ (ppb)	R^2	Carry over (%)	Matrix effect (%)		
									LQC	MQC	HQC
Acebutolol	0.25	1	3	100	400	600	0.9901	9.61	-7.98	-6.53	-10.49
Amlodipine	5	10	30	200	600	800	0.9935	16.13	-5.18	-1.62	-3.13
Aspirin	10	20	60	160	800	1000	0.9954	7.82	-12.88	-10.00	-6.26
Atenolol	5	20	60	200	800	1000	0.9907	3.83	12.71	0.76	-4.83
Atorvastatin	5	10	30	200	600	800	0.9935	6.67	18.06	5.38	-7.49
Bendro-flumethiazide	1	2	6	120	600	800	0.9958	6.38	0.61	7.28	3.03
Betaxolol	5	10	30	200	600	600	0.9931	12.51	13.16	0.43	9.94
Bevantolol	0.25	0.5	1.5	50	100	200	0.9934	19.48	-0.92	-21.12	-30.43
Bisoprolol	0.5	2	6	100	400	600	0.9915	8.43	-18.73	13.11	18.92
Captopril	1	2	6	100	400	600	0.9977	0.00	5.69	9.61	-14.08
Carvedilol	10	30	90	200	800	1000	0.9913	4.14	-42.68	-5.16	-3.95
Celiprolol	0.1	0.25	0.75	20	100	100	0.9870	13.04	10.74	7.56	7.02
Clonidine	0.5	2	6	100	400	600	0.9958	10.02	-0.47	7.50	-7.09
Clopidogrel	1	2	6	100	400	600	0.9966	12.72	-4.35	-19.91	-19.68
Diltiazem	0.25	1	3	100	400	600	0.9956	13.32	-1.45	-1.85	9.88
Doxazosin	1	5	15	100	400	800	0.9956	11.11	-10.33	-13.86	-11.55
Enalapril	0.1	0.5	1.5	50	400	600	0.9965	4.19	-13.18	-14.97	14.43
Fluvastatin	10	20	60	200	800	1000	0.9901	0.00	0.74	0.13	14.21
Furosemide	5	10	30	160	800	1000	0.9974	16.49	-12.88	-13.62	-7.21
HCTZ	50	100	300	800	4000	5000	0.9912	1.89	-13.53	0.22	-12.83
Indapamide	2	5	15	100	400	600	0.9899	16.21	10.72	-5.14	-8.89
Irbesartan	0.25	0.5	1.5	50	400	600	0.9949	17.56	-7.20	-14.24	-10.38
Labetalol	5	10	30	200	600	600	0.9908	12.62	16.89	8.10	18.69
Lisinopril	5	10	30	200	600	800	0.9920	2.83	-6.46	-1.34	8.39
Losartan	0.5	2	6	100	400	400	0.9872	14.83	4.31	-6.41	5.17
Lovastatin	2	10	30	200	600	600	0.9961	11.13	1.00	1.57	5.15
Metoprolol	0.25	0.5	1.5	50	400	400	0.9937	5.38	-3.26	-3.89	-15.46
Mevastatin	1	5	15	100	400	600	0.9936	13.12	18.40	11.00	12.93
Moxonidine	2	5	15	100	400	600	0.9933	5.34	12.78	-1.19	-4.26
Nadolol	0.5	1	3	100	400	400	0.9968	12.31	7.44	9.96	7.34
Nicotinic acid	50	100	300	800	1600	2000	0.9908	15.65	12.20	-44.78	-57.56
Olmesartan	0.25	1	3	100	400	600	0.9967	6.01	-3.24	-18.21	-11.91
Perindopril	0.25	1	3	100	400	600	0.9981	16.64	5.16	-1.73	12.64
Pindolol	0.1	0.25	0.75	20	100	100	0.9960	13.57	-2.38	18.02	4.80
Pitavastatin	0.25	0.5	1.5	50	100	200	0.9931	7.02	8.87	-6.10	1.03
Propranolol	2	10	30	200	600	600	0.9934	10.17	7.35	-4.53	-6.42
Ramipril	0.25	0.5	1.5	200	600	600	0.9969	17.20	-11.42	-0.23	-10.06
Rosuvastatin	5	10	30	200	600	600	0.9948	17.60	4.97	-19.92	-6.15
Spironolactone	10	30	90	200	800	1000	0.9938	9.76	-4.59	7.75	12.40
Telmisartan	1	5	15	100	400	600	0.9937	18.88	-17.32	1.78	-12.96
Terazosin	0.1	0.5	1.5	50	400	400	0.9922	10.84	-6.14	15.30	15.13
Triamterene	0.5	2	6	100	400	600	0.9944	6.14	-10.40	-11.75	0.57
Warfarin	2	5	15	100	400	600	0.9927	14.96	-13.62	-13.91	-12.69

Table 5. Precision, and accuracy results.

Compound	Intra-day (n = 5)								Inter-day (n = 3)							
	LLOQ		LQC		MQC		HQC		LLOQ		LQC		MQC		HQC	
	RE %	RSD %	RE %	RSD %	RE %	RSD %	RE %	RSD %	RE %	RSD %	RE %	RSD %	RE %	RSD %	RE %	RSD %
Acebutolol	109.14	19.73	113.00	4.77	106.72	7.09	103.24	7.07	103.82	18.21	110.75	8.65	107.22	7.27	95.68	5.80
Amlodipine	85.14	19.41	86.52	12.23	104.56	12.10	110.96	11.77	100.41	18.30	94.97	11.79	100.56	11.47	100.77	10.67
Aspirin	99.44	7.15	94.42	6.94	92.74	7.47	93.62	3.88	109.35	4.40	97.91	7.13	95.63	7.38	91.31	4.05
Atenolol	103.84	7.81	112.00	2.45	98.72	5.08	105.60	5.46	112.68	9.75	106.42	7.44	99.69	3.83	98.05	4.87
Atorvastatin	118.74	15.20	102.74	7.26	90.20	6.32	95.30	2.67	119.57	14.73	106.03	8.70	94.57	7.57	94.09	2.69
Bendroflu-methiazide	110.94	8.80	101.66	14.41	93.74	6.80	93.3	3.50	105.62	12.12	97.23	10.98	94.66	6.77	94.40	6.34
Betaxolol	104.24	7.82	108.80	6.08	92.54	3.14	85.94	5.07	101.95	9.30	106.73	8.01	100.41	6.16	86.89	4.14
Bevantolol	115.00	13.00	112.80	5.59	100.24	11.91	89.74	2.94	113.87	14.28	114.27	8.50	101.71	10.21	90.29	8.30
Bisoprolol	103.14	15.87	97.48	12.50	107.02	7.27	105.60	1.44	108.11	13.75	103.43	10.04	100.85	7.84	104.57	5.04
Captopril	115.90	12.26	113.02	14.78	113.80	1.44	108.60	3.10	112.72	9.99	106.93	12.96	105.21	6.63	94.90	7.23
Carvedilol	94.90	10.68	85.14	5.12	100.00	12.76	93.60	5.85	101.09	8.07	104.59	5.71	105.60	7.83	105.67	7.19
Celiprolol	119.78	17.80	88.14	14.68	101.88	10.82	107.06	7.16	119.21	18.54	91.87	14.13	99.16	11.26	101.78	9.43
Clonidine	104.80	11.99	102.78	12.51	95.08	8.49	92.72	3.34	111.92	12.39	108.41	9.33	104.99	10.49	93.94	4.88
Clopidogrel	91.68	9.18	99.26	11.61	97.68	11.96	113.40	3.61	107.71	13.25	101.30	10.83	96.75	9.14	113.15	5.29
Diltiazem	95.26	15.01	86.90	14.04	105.12	7.52	107.20	1.79	102.51	14.31	100.30	11.03	106.70	5.80	108.27	5.78
Doxazosin	111.94	14.83	102.26	12.46	105.46	6.60	102.16	3.52	114.63	13.95	101.13	10.36	102.07	4.70	99.77	5.21
Enalapril	115.42	19.13	97.92	9.15	90.40	6.75	88.74	4.80	109.75	16.75	98.73	13.39	94.93	6.58	98.92	4.32
Fluvastatin	118.00	14.43	112.36	13.63	95.26	13.36	89.32	5.44	118.14	15.24	111.33	11.30	94.49	9.20	96.17	6.10
Furosemide	96.92	10.86	96.14	1.48	97.38	6.31	105.6	2.28	98.81	11.48	97.41	5.06	93.48	6.87	95.96	5.23
HCTZ	106.66	12.31	99.42	10.68	104.12	8.12	96.06	4.88	101.85	11.84	99.82	9.88	100.74	7.66	94.09	6.24
Indapamide	117.96	19.36	104.58	8.06	106.88	7.32	106.60	3.15	112.88	15.47	109.96	12.17	103.27	6.65	109.87	3.70
Irbesartan	109.10	8.01	108.12	8.80	113.80	6.73	110.60	4.17	98.36	12.70	93.83	10.99	104.66	4.56	101.31	4.71
Labetalol	84.54	14.45	89.32	14.56	89.42	11.09	85.74	8.03	101.13	13.84	97.31	9.84	105.47	6.86	97.91	7.00
Lisinopril	115.98	10.74	109.40	2.93	108.00	4.86	115.00	7.22	108.77	9.93	102.10	5.42	105.51	7.88	103.81	7.30
Losartan	108.38	7.17	107.66	12.45	108.58	14.95	96.82	9.70	109.56	13.49	105.38	12.19	100.44	12.61	90.41	8.84
Lovastatin	111.26	17.11	85.66	11.34	97.60	8.56	106.00	14.82	102.61	17.25	94.25	9.48	89.87	11.16	107.91	13.77
Metoprolol	112.74	19.22	102.78	14.11	95.98	7.97	99.72	4.07	114.42	16.96	96.15	11.51	101.89	8.82	98.80	6.69
Mevastatin	113.40	12.16	98.14	10.63	95.84	10.60	106.08	10.60	108.06	13.58	96.35	11.14	93.77	11.88	102.53	11.65
Moxonidine	119.52	19.19	104.56	11.34	109.20	2.37	95.72	5.58	113.79	17.27	106.43	13.25	110.47	5.95	99.72	6.78
Nadolol	104.32	8.69	98.72	4.43	98.82	2.48	95.50	5.70	104.85	16.13	105.16	9.59	102.77	6.15	91.91	4.49
Nicotinic acid	104.44	13.59	94.92	9.70	108.80	5.41	100.26	13.25	101.26	12.88	96.92	9.48	97.55	8.69	99.60	8.64
Olmesartan	118.42	17.36	109.70	9.60	105.76	13.66	108.50	6.11	109.29	14.26	101.71	10.30	98.26	13.28	97.63	10.57
Perindopril	107.62	19.75	115.00	5.18	114.12	13.57	114.00	3.72	100.09	16.48	112.55	9.16	107.55	9.45	111.73	6.62
Pindolol	98.28	14.65	101.82	6.41	103.04	8.73	86.38	4.30	109.19	17.40	104.23	10.24	99.57	6.80	86.43	3.24
Pitavastatin	117.84	18.58	105.30	9.47	112.48	8.72	109.16	9.64	103.21	17.75	94.78	12.47	100.48	8.54	93.23	6.90
Propranolol	101.68	8.22	110.86	12.11	94.46	13.23	85.44	5.82	110.76	8.99	111.38	11.18	100.39	12.07	87.25	9.16
Ramipril	112.74	18.58	106.56	8.36	92.82	9.22	90.24	7.04	106.27	13.47	108.06	11.57	99.47	6.45	97.21	6.61
Rosuvastatin	93.72	12.37	90.04	14.54	101.16	5.99	112.60	4.33	107.57	8.85	103.41	11.61	96.65	6.74	112.13	4.53
Spirono-lactone	110.14	13.57	111.52	6.69	93.36	10.21	93.98	7.81	109.89	12.02	106.97	5.64	97.33	10.36	93.37	7.69
Telmisartan	117.46	11.31	106.48	13.41	102.00	13.08	108.32	9.16	116.09	14.97	100.79	11.85	100.95	13.13	101.43	12.66
Terazosin	98.64	19.87	100.58	15.46	103.44	6.46	103.62	6.53	111.46	14.96	97.79	10.72	102.49	8.89	102.45	7.03
Triamterene	92.22	7.59	103.72	10.22	105.08	7.16	89.30	7.56	107.81	8.05	107.74	9.18	107.99	8.80	95.63	6.35
Warfarin	89.48	17.74	97.20	14.12	93.76	8.16	107.20	2.67	91.45	14.93	99.17	12.87	101.35	5.92	100.69	3.43

Table 6. Comparison with related literatures.

No	Drugs	LOQ concentration (ppb)	
		Developed method	References
1	Amlodipine	10	25[14]
2	Atenolol	20	10[14]
3	Atorvastatin	10	1[16]
4	Bendroflumethiazide	2	10[14]
5	Bisoprolol	2	25[14]
6	Diltiazem	1	25[14]
7	Doxazosin	5	10[14]
8	Enalapril	1	1[14]
9	Fluvastatin	20	1[16]
10	Furosemide	10	10[14]
11	HCTZ	100	10[14]
12	Indapamide	5	10[14]
13	Irbesartan	0.5	1[14]
14	Labetalol	10	1[14]
15	Lisinopril	10	1[14]
16	Losartan	2	1[14]
17	Lovastatin	10	2[16]
18	Metoprolol	0.5	25[14]
19	Mevastatin	5	5[16]
20	Moxonidine	5	10[14]
21	Perindopril	1	0.5[14]
22	Pitavastatin	0.5	1[16]
23	Ramipril	0.5	1[14]
24	Rosuvastatin	10	1[16]

Discussion

A quick, cost-effective, and specific "dilute-and-shoot" LC–MS/MS method with minimal sample preparation process was investigated and validated for the determination of 43 prescribed antihypertensive and related drugs in human urine. The optimal mass spectrometric and chromatographic parameters were investigated by applying experimental design approach. The validation results indicated that this screening LC-MS/MS method was specific, reproducible, and sensitive with the limit of detection from 0.1 to 50.0 μg/L. For now, this dilute-and-shoot LC–MS/MS method has simultaneously screened a largest number of hypertensive and related drugs in human urine. In comparison with other related literatures, of the 24 drugs compared, 11 were improved the sensitivity and 10 had higher concentration of detection (Table 6). The less sensitivity of these compounds could be due to the simultaneously screening a larger number of analytes in different structures. The assay could be optimized for concurrently analysis 43 drugs but difficult to obtain the best solution for each compound. In particular, 4 of 10 less sensitive drugs belong statin group, which has a more specialized dilute-and-shoot LC–MS/MS method developed by Jang et al. 2018.[16]

Future expansion of the assay could include the addition of drug metabolites, because some drugs have short half-life as well as are metabolised and excreted as metabolites in urine, such as spironolactone, aspirin, ramipril, or fluvastatin. The assay also could be applied to the analysis of actual urine samples to validate its clinical effectiveness in further experiments.

Conclusions

In conclusion, the developed method could be a promising approach for screening the presence of prescribed cardiovascular drugs in human urine.

Supporting Information

Supporting information is available at https://drive.google.com/file/d/1QHBrI7yTj0MhxCK1U-8tcBhLXTn__uGv/view?usp=sharing.

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