Different Mobile Phases and Elution Programs were Optimized to Separate seven Basic Pharmaceuticals using LC-QTOF/MS

Fouad Fadhil Al-Qaim*, Md Pauzi Abdullah, Mohamed Rozali Bin Othman, Zuriati Binti Zakeria and Jalifa Latip.

***Abstract*—**Analysis of pharmaceuticals is very important, because these compounds have broad spectrum of chemicals and consider environmental emerging issue, so in this article several mobile phases and elution programs were optimized to separate 7 pharmaceutically active compounds with different therapeutic classes of pharmaceuticals using LC-QTOF/MS. A simple, reproducible and sensitive method for the separation of these compounds were provided using LC-QTOF/MS by gradient elution with a flow rate of 0.3 ml/min. The retention factor, selectivity, tailing factor and resolution were calculated for all compounds. Linearity for all compounds was satisfied in terms of R^2>0.99.Limit of detection and limit of quantitation for all compounds were (0.9-5 µg/l) and (3.2-16.6 µg/l) respectively.

***Keywords* —**Basic Pharmaceuticals, Retention Factor, LC-QTOF/MS, Tailng Factor, Resolution.

I. Introduction

PRAZOSIN 2- [4- (2- furoyl) piperazin- 1- yl]- 6 ,7 - dimethoxyquinazolin - 4- amine piperazine used to treat high blood pressure. Caffeine 1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione3,7-dihydro-1,3,7-trimethyl-1*H*-purine-2, 6-dioneis Caffeine is found in varying quantities in the seeds, leaves, and fruit of some plants, where it acts as a natural pesticide that paralyzes and kills certain insects feeding on the plants. Caffeine acts as a central nervous system stimulant.

Nifedipine 3,5-dimethyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate is a dihydropyridine calcium channel blocker. Its main use is antihypertensive. Simvastatin (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-{2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl] ethyl }-3, 7- dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate It is a member of the statin class of pharmaceuticals. used to control elevated cholesterol, or hypercholesterolemia. Carbamazepine 5*H*-dibenzo[*b*,*f*] azepine-5-carboxamide is an anticonvulsant .

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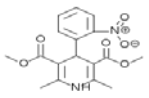
Levonorgestrel 13- ethyl- 17- ethynyl- 17-hydroxy- 1, 2, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17-tetradecahydrocyclopenta[a] phenanthren-3-one used as an active ingredient in some hormonal contraceptives. Enalapril 2*S*) - 1- [(2*S*)- 2- {[(2*S*)- 1- ethoxy-1-oxo-4-phenylbutan-2-yl]amino}propanoyl] pyrrolidine-2-carboxylic acid used in the treatment of hypertension [1] .

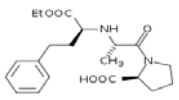
There are several analytical methods that used to analysis of different classes of pharmaceuticals in aquatic environment have been described in the literature, the most widely used technique was LC-MS/MS [2-4]. UPLC-TOF/MS technique was used to detect different classes of pharmaceuticals in waste water treatment plants effluents (WWTPs) and also in influents [5]. Different methods were used to determine several pharmaceuticals in different matrices, human plasma, urine, cocoa and pharmaceutical creams [6- 10].

Our objective in this article was to develop an RP-LC-QTOF/MS method for separation of caffeine (CAFF), enalapril (ENA), simvastatin (SIM), carbamazeoine (CARB), prazosin (PRAZ), levonorgestrel (LEVO) and nifedipine (NIF) (See TABLE I) as a mixture with best S/N and retention time.

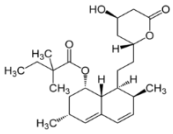
TABLE I
CHEMICAL STRUCTURES OF SEVEN BASIC PHARMACEUTICALLY ACTIVE COMPOUNDS.



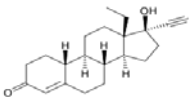
Nifedipine



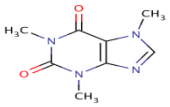
Enalapril



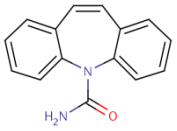
Simvastatin



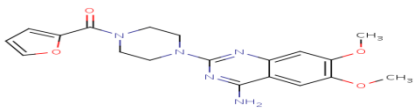
Levonorgestrel



Caffeine



Carbamazepine



prazosin

II. Experimental Part

A. Chemicals

Drug standards for nifedipine (CAS: 21829-25-4) , enalapril (CAS: 76095-16-4), prazosin (CAS: 19237-84-4), caffeine(CAS: 58-08-2), levonorgestrel (CAS: 797-63-7), carbamazepine (CAS: 298-46-4) and simvastatine (CAS: 79902-63-9) were obtained from Sigma Aldrich. Deionised Distilled Water from ALIR. HPLC-grade Methanol (Merck-106007), HPLC-grade acetonitrile (Merck- 100030), Formic acid (Merck-100264) were used.

B. LC-QTOF/MS Instrument

Separation of analytes was carried out using HPLC, Dionex Ultimate 3000/LC 09115047.

Identification of analytes were carried out using micro QTOF, the chromatographic separation was performed on a 250mmx 2.1mm Phenomenex C_{18} 5 micron.

The injection volume was 20 µL. Compounds analyzed in the positive ion (PI) mode were eluted off the column with several elution programs and mobile phase at 300 µL/min to get best separation with best S/N. The QTOF instrument was operated in wide m/z 50-1000(PI). Set nebulizer 2 bar, set dry heater 190 C^0 , set dry gas 7 l/min set capillary 4000v, set end plate offset -500 V and set collision Cell RF 250 Vpp.

C. Stock and Standard Solutions

Individually stock standard solutions were prepared in methanol and stored at -18C.

A mixture of all pharmaceutical standards was prepared by appropriate dilution of individual stock solutions. Further dilutions of this mixture were prepared in methanol-deionised water (25:75, v/v) before each analytical run and were used as working standard solutions.

Calibration

Mixed standard solutions containing caffeine (10-200 μg/l), prazosin (10-200 μg/l), enalapril-maleate (10-300 μg/l), carbamazepine (10-300 μg/l), nifidepine (10-200 μg/l), levonorgestrol (10-300 μg/l) and simvastatin (10-300 μg/l) were prepared in methanol and further dilution with 25% methanol in deionized water..

Four replications 20 μl injections were made for each standard solution to see the repeatability of the detector response (peak area) at each concentration level. The peak area of each drug was plotted against the concentration to obtain the calibration graph. The four concentrations of each compound were subjected to regression analysis to calculate the calibration equation and correlation coefficients (Squared-R).

III. Result and Discussion

A. Selection mobile phase and elution programs

Mobile phase and elution program play an important role to separate organic compounds well. In this work several experiments done using different elution programs(See TABLE II , III) and mobile phases to get the best separation and S/N ratio. However, we select one elution program and one mobile phase to evaluate chromatographic parameters (retention factor, selectivity, sensitivity, resolution and tailing factor).The selection of elution program and mobile phase was based on results of S/N for all pharmaceuticals (See TABLE IV).

TABLE II
SEVERAL ELUTION PROGRAMS TO MAKE OPTIMIZATION.

Program	Gradient Program								
PROG(1)	**TIME (MIN)**	**0**	**3**	**6**	**11**	**11.1**	**16.1**		
	B%	**5**	**60**	**97**	**97**	**5**	**5**		
PROG (2)	TIME (MIN)	0	0.5	6	9	12	13	18	
	B%	5	5	60	97	97	5	5	
PROG(3)	TIME (MIN)	0	2	4	5	10	10.1	15.1	
	B%	5	70	70	97	97	5	5	
PROG(4)	TIME (MIN)	0	1	5	9	13	13.1	18.1	
	B%	5	40	80	100	100	5	5	
PROG(5)	TIME (MIN)	0	7	13	13.1	18.1			
	B%	10	100	100	10	10			
PROG(6)	TIME (MIN)	0	3	5	8	13	13.1	18.1	
	B%	5	70	70	97	97	5	5	
PROG(7)	TIME (MIN)	0	1	11	17	24	25	30	
	B%	5	5	60	90	90	5	5	
PROG(8)	TIME (MIN)	0	0.5	5	8	10	13	14	19
	B%	5	5	70	70	97	97	5	5
PROG(9)	TIME (MIN)	0	8	10	13	14	19		
	B%	5	90	95	95	5	5		

B. Method development

The mobile phase was chosen after several trials with different percentages of methanol, acetonitrile, deionised water and formic acid solutions in various proportions.

A mobile phase consisting of *A: 0.1% formic acid in deionised water and B: 75:25 acetonitrile : methanol v/v*, was selected to achieve maximum separation and best S/N.

Flow rates 0.2 and 0.3 ml/min were studied. A flow rate of 0.3 ml/min gave an optimal signal to noise ratio with a

reasonable separation time. Using a reversed-phase C18 column, the retention times for caffeine, prazosin, enalapril, carbamazepine, nifidipine, levonorgestrel

and simvastatine were observed to be 6.6 min, 7.0 min, 7.6 min, 8.6 min, 9.3 min, 10.1 min and 11.5 min respectively. Total time of analysis was 16.1 min (See Fig I).

TABLE III
ELUTION PROGRAM AND MOBILE PHASE TO SEPARATE BASIC PHARMACEUTICALS

Elution Program							
Time(min)	0	3	6	11	11.1	16.1	
B%	5	60	97	97	5	5	
Mobile Phase							
A	0.1% FA in Deionised Water.						
B	75% ACN:MeOH (v/v).						
Signal/Noise							
Compound	Caff	Praz	Ena	Carb	Nif	Levo	Sim
RT(min.)	6.6	7	7.7	8.6	9.3	10.1	11.5
S/N	68.9	155.8	135.2	221.2	211.7	105.4	136.8

TABLE IV
SEVERAL MOBILE PHASE TO MAKE OPTIMIZATION.

MOBILE PHASE	CONTENTS
M.P (1)	A: 0.1% FA in Deionised Water. B: 0.1% FA in 2: 1 ACN:MeOH (v/v).
M.P. (2)	A: 0.1% FA in Deionised Water. B: 69% ACN:MeOH (v/v).
M.P. (3)	**A: 0.1% FA in Deionised Water.** **B: 75% ACN:MeOH (v/v).**
M.P. (4) OVERLAP	A: 0.1% FA in 1:1:1 ACN:MeOH:H2O (v/v/v). B: 40% ACN:MeOH (v/v).
M.P. (5)	A: 0.1% FA in Deionised Water. B: 0.1% FA in 100% ACN.
M.P. (6)	A: 0.1% FA in Deionised Water. B: 2:1 ACN:MeOH (v/v).
M.P. (7)	A: 0.1% FA in Deionised Water. B: 100% ACN.
M.P. (8)	A: 0.1% FA in Deionised Water. B: 40% ACN/MeOH (v/v).
M.P. (9)	A: 0.1% FA in Deionised Water. B: 0.02% FA in 2: 1 ACN:MeOH.
M.P. (10)	A: 0.1% FA in Deionised Water. B: 0.04% FA in 2:1 ACN:MeOH (v/v).
M.P. (11)	A: 0.1% FA in Deionised Water. B: 67% ACN:MeOH (v/v).
M.P. (12)	A: 0.1% FA in Deionised Water. B: 67.5% ACN:MeOH (v/v).

C. Suitability of the method

The chromatographic parameters such as resolution ,retention factor, selectivity and peak asymmetry were satisfactory for these compounds (See TABLE V). The calculated resolution values between each peak-pair were no less than 1.7 and the selectivity was not less than 1.08.

Retention factor values were found to be 2.27, 2.47, 2.76, 3.26, 3.6, 4.00 and 4.69 for caffeine, prazosin, enalapril, carbamazepine, nifidipine, levonorgestrol, and simvastatine respectively.

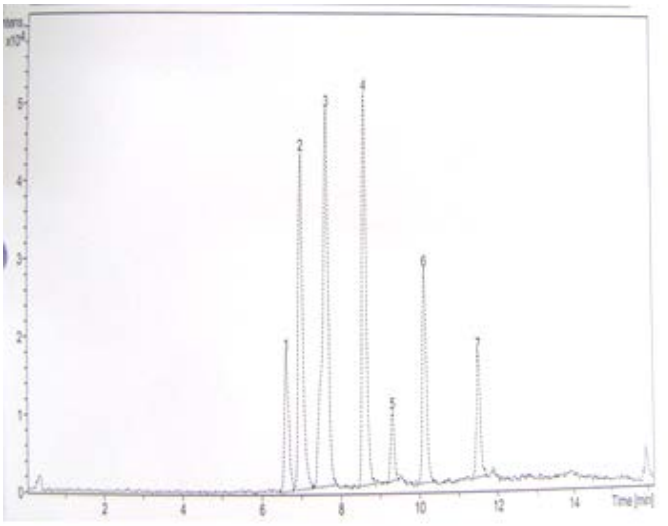


Fig.1 Chromatogram of the mixture of caffeine (1), prazosin (2), enalapril (3), carbamazepine (4), nifidipine (5), levonorgestrol (6) and simvastatine (7) developed by the LC –QTOF/MS method.

TABLE V
CHROMATOGRAPHIC PARAMETERS FOR ALL BASIC PHARMACEUTICALS.

compounds	parametrs			
	K, n=4,mean	R	α n=4,mean	Tf
Caff	2.27 , 0*			1.667
Praz	2.47 , 0	1.767	1.09 , 0*	1.7
Enal	2.76 , 0	2.162	1.12 , 0	0.967
Carb	3.26 , 0	3.767	1.18 , 0	2.125
Nif	3.60 , 0	3.465	1.10 , 0	1.214
Levo	4.00 , 0	4.113	1.11 , 0	1.875
Sim	4.69 , 0	7.143	1.17 , 0	1.4

* RSD% = [S.D/mean] × 100%

D. Precision

The precision of the method (within-day variations of replicate determinations) was checked by injecting caffeine, prazosin, enalapril, carbamazepine, nifidipine, levonorgestrol, and simvastatine as a mixture 4 times at the 200 ppb. The precision of the method, expressed as the RSD % at 200 ppb, was presented in TABLE V1.

TABLE V1
PRECISION OF DEVELOPED METHOD AT 200 PPB.

comp	parametrs			
	Tr, n=4 Mean ± SD	RSD %	Peak Area, n=4 Mean ± SD	RSD %
Caff	6.6 ± 0	0	109681.5±3852.798	3.51
Praz	7.0 ± 0	0	298559.8±12994.87	4.35
Enal	7.6 ± 0	0	432127.3±22429.23	5.19
Carb	8.6 ± 0	0	370115±6021.348	1.62
Nif	9.3 ± 0	0	70758.75±4913.078	6.94
Levo	10.1 ± 0	0	201368.5±10787.24	5.35
Sim	11.5± 0	0	183076.3±7574.472	4.13

Linearity

Calibration curves were prepared for each compound as a mixture in a 25%MeOH in deionized water by plotting the average total peak area versus the analyte concentration.

Figure 3 displays the plots of the calibration curves and correlation coefficients R^2 which are higher than 0.99 (Fig 2).

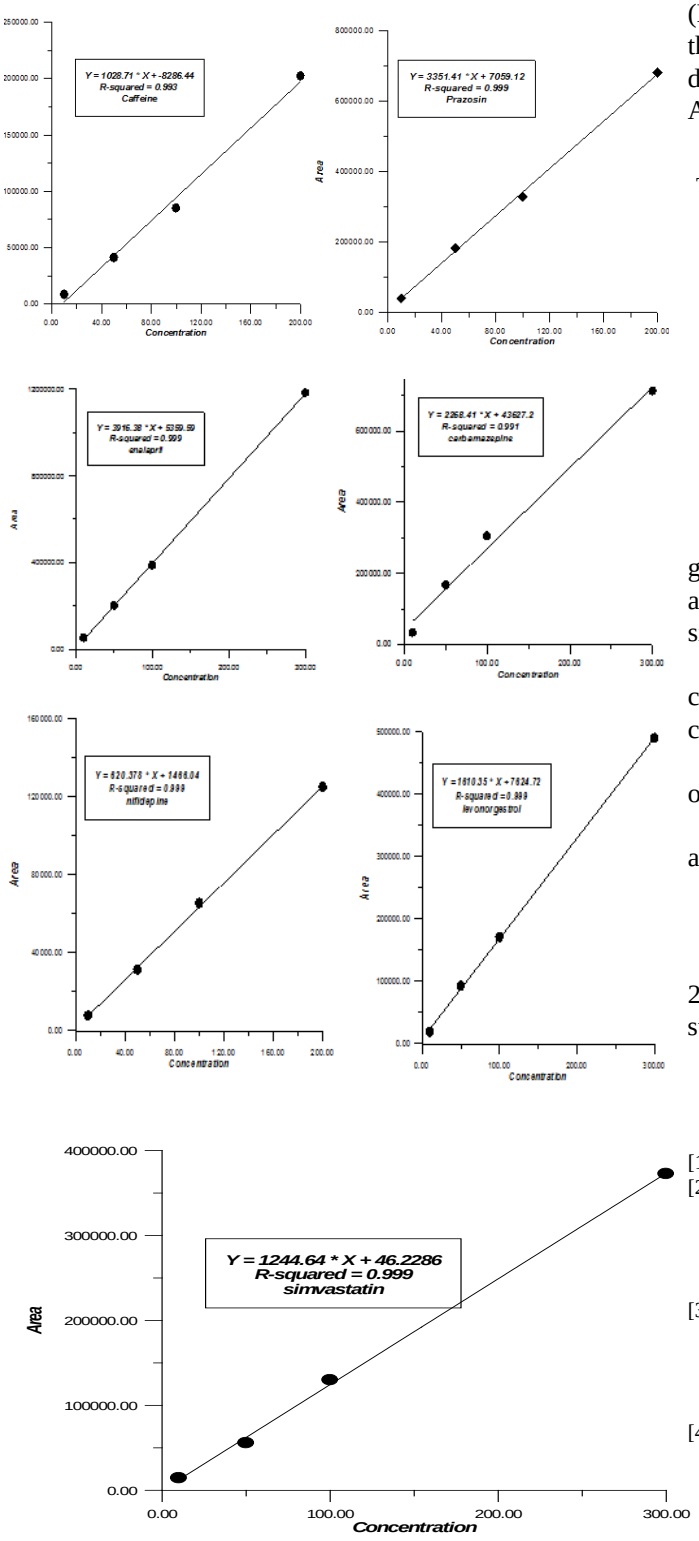


Fig. 2 Calibration curves of seven compounds caffeine, prazosin, enalapril, carbamazepine, enalapril, levonorgestrol and simvastatin.

Limits of Detection and Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were determined by injecting progressive dilutions of the standard solution as a mixture. The LOD and LOQ were defined as a signal/noise ratio of 3:1 and 10:1, respectively. All values of LOD and LOQ were presented in Table VII.

TABLE VII: DETECTION LIMIT (LOD) AND QUANTIFICATION LIMIT (LOQ) OF SEVEN COMPOUNDS.

Compound	LOD (ng/ml)	LOQ (ng/ml)
Caffeine	4.3	14.3
Prazosin	0.9	3.2
Enalapril	1.1	3.5
Carbamazepine	1.1	3.7
Nifidepine	5	16.6
Levonorgestrol	1.8	5.9
Simvastatin	2.8	9.4

IV. CONCLUSION

Mobile phase and elution program were very necessary to get good separation and signal to noise ratio. Using formic acid in mobile phase was good to enhancement the mass signal of compounds in order to get good result.

Limit of detection and limit of quantification for compounds were in ng/ml, so it was possible to analysis compounds in aquatic environment.

The linearity of all compounds was very satisfied in terms of $R^2 > 0.99$.

We advise to continue to analysis these pharmaceutically active compounds in rivers and waste water treatment plants.

ACKNOWLEDGMENTS

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REFERENCES

[1] http://en.wikipedia.org/wiki/Main_Page

[2] N.A.Al-Odaini, M.P.Zakaria, M.I.Yaziz and S.Surif. "Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid –phase extraction and liquid chromatography-tandem mass spectrometry". Journal of chromatography A,1217 , 2010, 6791-6806.

[3] M.D.Hernando, M.Petrovic, A.R.Fernandez-Alba and D.Barcelo. "Analysis by liquid chromatography –electrospray ionization tandem mass spectrometry and acute toxicity evaluation for β-blockers and lipid –regulating agents in wastewater samples". Journal of chromatography A, 1046, 2004, 133-140.

[4] M.J.Gomez, M.Petrovic, A.R.Fernandez-Alba, D.Barcelo.."Determination of pharmaceuticals of various therapeutic classes by solid-phase extraction and liquid chromatography-tandem mass spectrometry analysis in hospital effluent wastewaters". Journal of chromatography A,1114, 2006, 224-233.

[5] M.D.Hernando, M.Mezcua, A.R.Fernandez-Alba, D.Barcelo." Environmental risk assessment of pharmaceutical residues in wastewater effluents,surface waters and sediments". Talanta, 69, 2006, 334-342.

[6] E.P.C.Lai and S.Y.Feng.."Solid phase extraction –Non-aqueous capillary electrophoresis for determination of metformin , phenformin and glyburide in human plasmas". Journal of chromatography B, 843 , 2006, 94-99.

[7] M.Xu and J.Song." Polarography determination of lovastatin in the presence of H2O2 in pharmaceuticals, human urine and serum". Microchim. Acta, 148, 2004, 183-189.

[8] M.R.Brunetto, L.Gutierrez, Y.Delgado, M.Gallignani, A.Zambrano, A.Gomez, G.Ramos and C.Romero. "Determination of theobromine, theophyllin and caffeine in cocoa samples by a HPLC method with on-line sample cleanup in a switching-columnsystem". Food Chemistry, 100 , 2007, 459-467.

[9] D.Bonazzi, V.Andrisano, R.Gatti and V. Cavrini. "Analysis of pharmaceutical creams: a useful approach based on solid-phase extraction (SPE) and UV spectrophotometry". Journal of Pharmaceutical and Biomedical Analysis. 13, 1995, 1321-1329.

[10] M. Aqil, A. Ali, A. Ahad, Y. Sultana, A. K. Najmi, and N. Saha. "A Validated HPLC method for estimation of metoprolol in human plasma". Acta Chromatographica, 19, 2007., 130-140.