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Research Article

VALIDATED RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF AMLODIPINE BESYLATE, AND HYDROCHLOROTHIAZIDE IN BULK AND PHARMACEUTICAL FORMULATION

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ABSTRACT

Amlodipine Besylate and hydrochlorothiazide combination is a long acting calcium channel blockers used as anti-hypertensive and for the treatment of angina. The combination is planned to be introduced very soon in the market in the Sudan. It is of vital importance that a validated and very precise analytical method should be established for the quantification of the components of this drug combination. The present study describes a reliable reverse phase high performance liquid chromatographic RP-HPLC method that has been developed and validated for the simultaneous estimation of amlodipine besylate and hydrochlorothiazide in pharmaceutical formulation. The combination werefirstlyHPLC assayed and excellently resolved peaks were obtained via an RP – C_{18} column. The mobile phase (mixture of Buffer pH 3.0: Acetonitrile: Methanol) was pumped at a flow rate of 1.0 mL min^{-1} in the ratio of (500: 300: 350, v/v) and the eluents were monitored by a uv-detector set up at 240 nm. The retention time for amlodipine and hydrochlorothiazide was found to be 7.0 min and 3.0 min, respectively. Linearity was ascertained via linear calibration curves for both drugs (R^2= 0.9996 for amlopedinebesylate and 0.99912 for hydrochlorothiazide) within the concentration range of 2.0–48 µg ml^{-1} for amlodipine besylate, and10.0–120 µg ml^{-1} for hydrochlorothiazide. The method was statistically validated and RSD was found to be less than 2% indicating high degree of accuracy and precision of the proposed HPLC method. The percentage recoveries from the combined dosage form were between98.47% to 100.51% and98.12%to101.42%**.** The method is simple, rapid and of high degree of precision and accuracy. The method can, confidently, be applied and utilized in pharmaceutical quality control laboratories in routine analysis for determining amlodipine besylate, and hydrochlorothiazide in bulk and in pharmaceutical form.

Keywords: Amlodipine besylate, hydrochlorothiazide and RP – HPLC.

INTRODUCTION

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Amlodipine besylate AMB, 3-ethyl-5-methyl-2-[(2-aminoethoxymethyl]-4-(chloro-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate is a chiral calcium antagonist and it is a long acting calcium channel blocker used as an anti-hypertensive drug and also be used for the treatment of angina[1]. Hydrochlorothiazide, 6–chloro- 3,4–dihydro–7–sulfamoyl–2H–1,2,4–benzothia–diazine – 1, 1 –dioxide, is a thiazide diuretic[2]**.** The chemical structure of amlodipine besylate and hydrochlorothiazide. Figure 1and Figure 2, shown below**:**

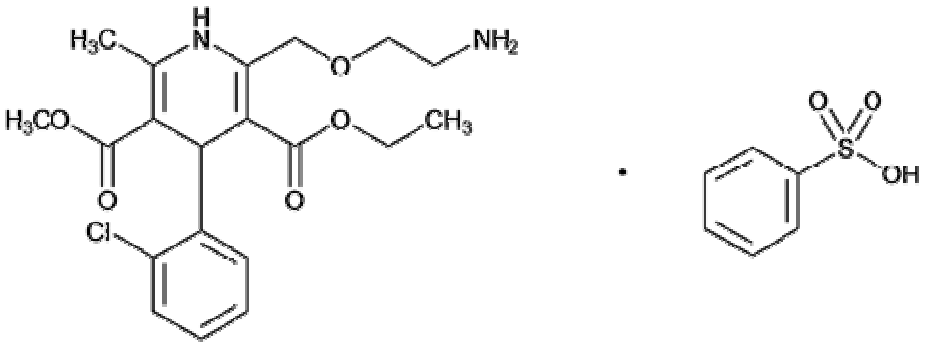


Fig. 1: Amlodipine besylate

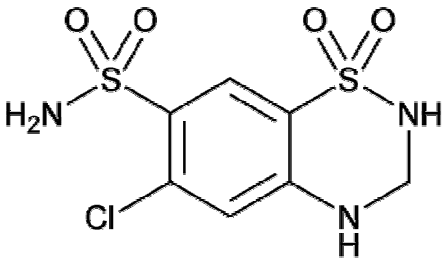


Fig. 2: Hydrochlorothiazide

Various analytical methods have been attempted and reported for the assay of AMB alone and quite few in combinations with other anti-hypertensive agents in pharmaceutical formulations. These include UV spectroscopy[2-4], high performance liquid chromatography[5,6], LC-MS and LC-MS/MS[5,6]. Many analytical methods were reported for the analysis of (HCT) alone and in combination with other drugs by stability indicating methods and had been determined in plasma[5]. Amlodipine and hydrochlorothiazide are official in USP[4]. The chemical structure of amlodipine and hydrochlorothiazide.Search in literature revealed that there is no available official method for the simultaneous determination for this drug combination. Moreover, based on the fact that, currently, HPLC-analytical tool and the procedures associated with it, specifically, RP–HPLC procedure have proved to be simple, accurate and of high degree of precision. Accordingly, the present study is an attempt to develop and validate an RP-HPLC-procedure for simultaneous estimation of amlodipine and hydrochlorothiazide in bulk and in pharmaceutical preparations. This research study is representing one of our research group intentions and interests to implement research investigations and studies dealing with the constitution of drug combinations marketed in the Sudan.

MATERIALS AND METHODS

MATERIALS

All analytical runs were performed in a HPLC-Shimadzu (Japan) chromatograph equipped with an LC – 20ABsolvent delivery system, a universal loop injector (SIL20A) of injection capacity of 100 µl, and an SPD – 20 AV UV–Visible detector set at 240 nm. The instrument was equipped with a GL SCIENCES C18 column of the dimensions (250mm x 4.6mm i.d., 5 µm particle size). An isocratic elution was adopted using a mixture of Buffer pH 3.0: Acetonitrile: Methanol (500: 300: 350, v/v), as a mobile phase. Flow rate of mobile phase was adjusted to 1.0 ml.min^{-1} and injection volume was 50 µl at 40°C temperature. Normal run time was chosen as 15minutes. The equipment was controlled by a PC work station with Win Chrome Software. Analytically pure samples of amlodipine besylate and hydrochlorothiazide were procured from Azal Pharmaceutical Company, Khartoum north, Sudan as a gift and used as working standards. methanol of HPLC grade from ROMIL, Acetonitrile of HPLC grade from Chemical lab (CL), triethyl Amine of HPLC Grade from Sharlau, all other reagents are of analytical grade.

METHODS

Preparation of standard solution

Amlodipine Besylate (20 mg) and hydrochlorothiaiazide (50 mg) working standard was, accurately, weighed and introduced in a 100ml volumetric flask. The contents were dissolved in the mobile phase (30 ml) and sonicated. The solution was made up to 100 ml by the mobile phase. 5 ml of this solution were mixed with the internal standard (5 ml). The solution was made up to 50 ml by the mobile phase in a 50 ml volumetric flask.

Preparation of buffer solution

Triethylamine (7 Ml) was added, with stirring, to water (800 mL). the pH of the resulting solution was adjusted to pH 3 through the drop wise addition of *ortho*-phosphoric acid. The solution was then diluted, with distilled water, to 1000 mL.

Preparation of sample solutions

The sample drug (20 tablets, 140 mg) was, accurately, weighed and crushed to a course powder. The powder constituted 5mg of amlodepine and 12.5 mg of hydrochlorothiazide. The powder was transferred to a 100ml volumetric flask. The mobile phase (70 ml) was added and the mixture was shaken for complete solution and then sonicated for around 10 minutes with occasional shaking. The mobile was added to mark to make up to 100ml solution. Aportion of this solution (20 ml) was made up by the mobile phase to 50 ml in another volumetric flask. The final solution was filtered through 0.45 µm GHP filter.

Preparation of the Test Solutions (50%, 100% and 150% Solutions)

Amlodepine Besylate WS (2.5 mg), hydroclorothiazide WS (6.3 mg) and the placebo (122.5 mg) were thoroughly mixed and transferred into a 100 ml volumetric flask and then dissolved in the mobile phase (70 ml), sonicated to ensure complete dissolution. After cooling the volume was made up to the mark by the addition of the appropriate amount of the mobile phase. 10 ml portion of this solution was diluted to 25 ml with mobile phase to afford a 50% solution.

In a similar manner, for the preparation of a 100% and 150% different amounts (weights) of the drug combination be considered. Amlodepine Besylate WS (2.5 mg), and hydroclorothiazide WS should be 5 mg and 7.5 mg for the former drug and 12.5 mg, 16.8 mg for the latter drug, respectively. The appropriate volumes be taken and diluted to afford these two percentages

Specificity preparations

Standard preparation

Amlodepinebesylate WS (20 mg) and hydrochlorothiazide WS (50 mg) were, accurately weighed, mixed, transferred into a 100ml volumetric flask and dissolved in the mobile phase (70 mL). The solution was sonicated for few minutes, then cooled and the volume was completed to the mark by the mobile phase. A volume (5 mls) of this solution was diluted to 50 mL by the mobile phase.

Test preparation solution

Amlodepine Besylate WS (5 mg), hydroclorothiazide WS (12.5 mg) and placebo (122.5mg) were accurately weighed and transferred into a 100 ml volumetric flask and a 70 mL of the mobile phase was added. The contents were thoroughly mixed and sonicated for few minutes. The solution was allowed to cool and the volume was completed to the mark by the mobile phase. 10 mL of this solution was diluted to 25 mL with the mobile phase**.**

Acid hydrolysis test (0.1N hydrochloric acid)

Amlodepine Besylate WS (5mg), hydroclorothiazide WS (12.5 mg) and of place bo (122.5 mg) were accurately weighed and transferred into a 100 ml volumetric flask. An aqueous hydrochloric acid (0.1N HCl, 5 mL) was added. The solution was allowed to stand for 2 hrs and about 70 mL of the mobile phase was added. The solution was then sonicated for few minutes, allowed to cool and the volume was made up to the mark with the mobile phase. A volume of 10 mL of this solution was diluted to 25 mL.The appropriate volume of this solution was injected in the HPLC-system and the chromatogram was studied and recorded.

Base hydrolysis (0.1N sodium hydroxide)

Amlodepine Besylate WS (5 mg), hydroclorothiazide WS (12.5 mg) and placebo (122.5 mg) were weighed accurately and transferred into a 100 mL volumetric flask. An aqueous solution of sodium hydroxide (0.1 N, 5 mL) was added and the solution was allowed to stand for 2 hrs. 70 Ml of the mobile phase was added and the contents of the flask were sonicated for few minutes, allowed to cool and the volume was made up to the mark by the mobile phase. 10 ml of this solution was diluted to 25 ml by the mobile phase.The appropriate volume of this solution was injected in the HPLC-system and the chromatogram was studied and recorded.

Hydrogen peroxide oxidation test

AmlodepineBesylate WS (5 mg), hydrochlorothiazide WS (12.5 mg) and placebo (122.5mg) were weighed accurately and transferred into a 100 ml volumetric flask. Hydrogen peroxide (5 mL, 30% solution) was added and the contents of the flask were allowed to stand for 2 hrs. 70 mL of the mobile phase was added and the contents were sonicated. It was then allowed to cool and the volume was made up to the mark by the mobile phase. 10 mls of this solution was diluted to 25 mls with the mobile phase.The appropriate volume of this solution was injected in the HPLC-system and the chromatogram was studied and recorded

Thermal stability test: Test preparation for Heat hydrolysis (at 80ºC for 72 hours)

Amlodepine besylate WS (5.0 mg), hydroclorothiazide WS (12.5 mg) and the placebo (122.5 mg) were weighed accurately, transferred into a 100 mL volumetric flask. It was then placed into a dry oven set at 80ºC and allowed for 72 hr. The solution was then transferred into a 100 mL volumetric flask and 70 mL of the mobile phase was added. The contents of the flask were sonicated and then allowed to cool. The volume was then made up to the mark with the mobile phase. 10 mL of this solution was diluted to 25 mL by the mobile phase. The appropriate volume of this solution was injected in the HPLC-system and the chromatogram was studied and recorded.

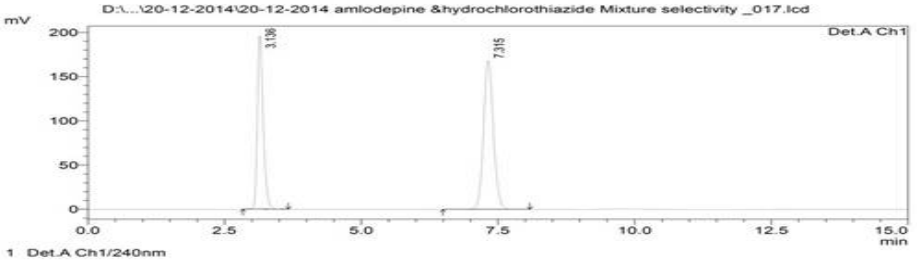
RESULTS AND DISCUSSION

The protocol adopted for the establishment the HPLC analytical procedure presented in this current work consisted of: choosing optimum HPLC-conditions and suitable mobile phase composition to achieve an excellent resolution of the individual working standards, amlopedine WS and hydrochlorothiazide WS drugs and thereafter the resolution of a 1:1 ratio by weight mixture of the two drugs. The second phase comprises HPLC determinations of ranges of concentration levels of each component drug working standards to establish linearity plots. The third phase involves the derivation and determination of the validation parameters associated with the results obtained in terms of linearity, accuracy, precision, coefficient of variation, reproducibility and specificity of the sample applications. The fourth phase is a preliminary attempt for the application of method in monitoring drug stability and the final phase is the statistical data study for the derivation of a number of validation parameters.

HPLC-Resolution of the Drug Combination

The HPLC-instrument employed in this work was a Shimadzu (Japan) Model ------equipped with a UV-detector being set at λ 240 nm and an RP-C18 Column. Other HPLC-conditions were presented in the Materials and Methods Section. The first organic solvent composition of the mobile phase was: buffer pH 3.00: methanol: acetonitrile 50: 35: 30 v/v, which has given good resolution but perturbed shapes of the peaks.

HPLC-runs have been performed in which the buffer was kept constant and the composition of the organic solvents varied. An excellent resolution and best peak shapes were reached when the mobile phase composition of (Buffer pH 3.0: Methanol: Acetonitrile 50:35:30v/v) was attempted. This solvent mixture was used to resolve the individual working standards amlopedine WC and hydrochlorothiazide WS drugs at similar concentrations affording a retention time of 3.148 min for amlopedine and 7.323 min for hydrochlorodiazide. Moreover, a 1:1 ratio combination of the two drugs mixture has shown an excellent resolution as shown below.



PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %
1	3.136	1459453	195869	40.844
2	7.315	2113802	168387	59.156
Total		3573255	364256	100.000

Fig. 3: Resolution of amlopedine WC and hydrochlorodiazide 1:1 mixture

It was observed that optimizing acetonitrile composition in the mobile phase was a determining factor in improving the resolution, maintaining good peak shape and minimizing the HPLC-run time. Accordingly, the following optimum mobile phase ratio: buffer pH 3.00: methanol: acetonitrile 50: 35:30 v/v, was reached after conducting a number of HPLC-trials involving varying volumes of acetonitriles versus fixed volumes of methanol and buffer. The components of the combination drug have been resolved without any interferences, Figure 1.accordingly, the fore-mentioned composition of the mobile phase has been used throughout the work at a flow rate of 1.00 ml/min.

Determination of the Linearity Parameter

The linearity parameter was determined by injecting a series of nine concentration levels within the range 0.004-0.048 µg/ml and 0.01-0.12 µg/ml, for each amlopedine WC and hydrochlorothiazide WC, respectively. The response of each of the two drugs was found to be linear within its investigation concentration range and the linear regression equation was y = 104171508.6 – 16646.067with a correlation coefficient 0.9996 for amlopedine and y = 26571745.2+ 78518.17499 with a correlation coefficient of 0.99912 for hydrchlorothiazide. The results obtained for both drugs have shown an excellent coefficient of variation and reproducibility, which was evident from the low relative standard deviation RSD ranging from 0.09 to 0.04 for amlopedine and 0.45 to 0.04 for hydrochlorodiazide see Table 1 and Table 2, below.

Table 1: Regression analysis data for Amlodipine besylate

	Level 01	Level 02	Level 03	Level 04	Level 05	Level 06	Level 07	Level 08	Level 09
	0.004	0.008	0.012	0.016	0.02	0.024	0.03	0.04	0.048
1st		861467		1678699	2053975	2493502		4253520	5004300
2nd	458157	861333	1275471	1677229	2065291	2488682	3116068	4245410	5005647
3rd	457232	861100	1275693	1677707	2061641	2488840	3117893	4245371	5004332
4th	457395	860314	1276235	1679733	2060919	2489458	3118295	4248303	5001487
5th	457429	860430	1274861	1679274	2061593	2489728	3116450	4251036	5000502
Average	457553	860929	1275565	1678528	2060684	2490042	3117177	4248728	5003254
RSD%	0.09	0.06	0.04	0.06	0.20	0.08	0.03	0.08	0.04

Table 2: Regression analysis data for Hydrochlorothiazide

	Level 01	Level 02	Level 03	Level 04	Level 05	Level 06	Level 07	Level 08	Level 09
Conc	0.01	0.02	0.03	0.04	0.05	0.06	0.075	0.1	0.12
1st		603793		1162369	1396690	1691162		2783060	3216341
2nd	308911	601942	887844	1161879	1399401	1694569	2086121	2775095	3215111
3rd	307433	602292	889174	1162848	1395347	1695049	2080131	2769000	3214238
4th	308832	602730	889384	1161789	1400579	1691337	2084405	2771940	3217354
5th	306008	602811	889331	1162917	1400307	1691161	2080012	2771539	3215342
Average	307796	602714	888933	1162360	1398465	1692656	2082667	2774127	3215677
RSD%	0.45	0.12	0.08	0.05	0.17	0.12	0.15	0.20	0.04

A linearity plot of concentration versus intensity (area under the peak) was established for each of the working standards, Figure (4) and Figure (5), respectively.

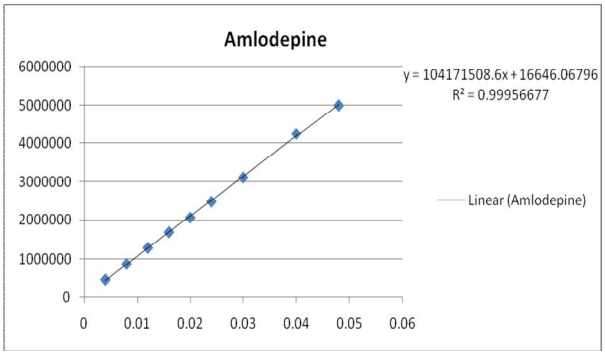


Fig. 4: Linearity plot of amlodipine besylate

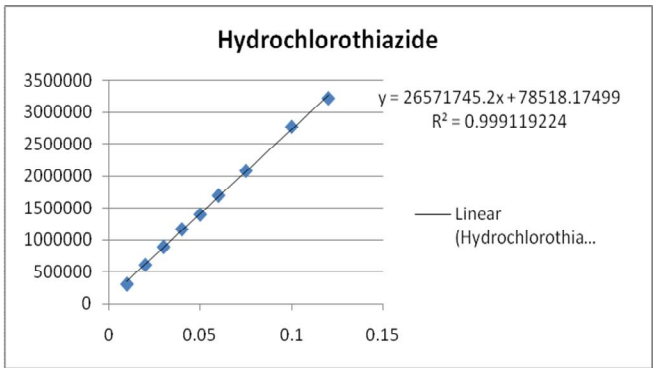


Fig. 5: Linearity plot of hydrochlorothiazide

Determination of Precision and Accuracy Parameters

The precision of the assay method was evaluated in terms of repeatability by carrying out six independent assays of test sample preparation and calculated the % RSD of assay (intraday). Intermediate precision of the method was checked by performing the same procedure on the different day (intraday) by another analyst under the same experimental conditions. The intermediate precision, which is less than 2.0%, is an evidence for the excellent repeatability of the results indicating that the method is of high precision. It is noteworthy, to mention that the repeatability parameter could be determined from the precision and accuracy, since all three parameters are inter-related.

Table 3: Intraday Precision for Amlopedine Besylate and Hydrochlorothiazide

Precision 1									
Amlodepine					**Hydrochlorothiazide**				
P		WC	average	Cliam	P		WC	average	Cliam
99.16		0.17	140	5	100.32		0.01	140	12.5
M.W. AmlodepineBesylate			567.05						
M.W. Amlodepine			408.88						
	STD1	STD2	Test1	test2		STD1	STD2	Test1	test2
wieght	20.5	20.1	140	140	wieght	50.6	49.9	140	140
Inj#01	2106337	2057397	2838384	2890719	Inj#01	1500506	1464655	1438537	1469486
Inj#02	2108852	2057230	2835887	2895586	Inj#02	1505230	1466544	1434897	1468034
Inj#03	2111299				Inj#03	1501055			
Inj#04	2108366				Inj#04	1508001			
Inj#05	2104841				Inj#05	1500101			
average	2107939	2057314	2837136	2893153	average	1502979	1465600	1436717	1468760
RSD	0.12	0.01	0.06	0.12	RSD	0.23	0.09	0.18	0.07
Agree	100.46	assay	98.47	100.42	Agree	101.13	assay	97.04	99.20
		average	99.45				average	98.12	
		RSD	1.38				RSD	1.56	

Table 4: Interday Precision for AmlopedineBesylate and hydrochlorothiazide

Precision 2									
Amlodepine					Hydrochlorothiazide				
P		WC	average	Cliam	P		WC	average	Cliam
99.16		0.17	140	5	100.32		0.01	140	12.5
M.W. Amlodepine Besylate			567.05						
M.W. Amlodepine			408.88						
	STD1	STD2	Test1	test2		STD1	STD2	Test1	test2
wieght	20.1	20.1	140	140	wieght	50.1	50	140	140
Inj#01	2074348	2065650	2935238	2881465	Inj#01	1465416	1449695	1482442	1466488
Inj#02	2074232	2066312	2923863	2885754	Inj#02	1463146	1456064	1475850	1478408
Inj#03	2074063				Inj#03	1459892			
Inj#04	2075102				Inj#04	1461488			
Inj#05	2074634				Inj#05	1463102			
average	2074476	2065981	2929551	2883610	average	1462609	1452880	1479146	1472448
RSD	0.02	0.02	0.27	0.11	RSD	0.14	0.31	0.32	0.57
Agree	100.41	assay	101.30	99.72	Agree	100.47	assay	101.65	101.19
		average	100.51				average	101.42	
		RSD	1.12				RSD	0.32	

The accuracy of the method was determined by recovery of spiked pre-analyzed sample formulation of the drug in triplicate sets of concentration levels: 50%, 100%, and 150%. The robustness of procedure was investigated to evaluate the influence of small but deliberate variations in the chromatographic conditions, such as changes in the flow rate [-+0.1ml\min], a change in the wavelength [+/- 2.0 nm] [and changes in the mobile phase composition 0.02 M ammonium acetate buffer 4.5 Acetonitrile (62:38and 58:42v/v) and using different lot of LC column]

Table 5: Percentage Recoveries Spiked AmlopedineBesylate

Accuracy Amlodepine besylate										
		50%			100%			150%		
	STD	T1	T2	T3	T1	T2	T3	T1	T2	T3
Wt	20	2.5	2.5	2.6	5	5.2	5	7.4	7.4	7.4
1st	2021595	987448	996008		2012570	2106483	2026410	3023346	3051648	
2nd	2022242	982196	998542	1071809	2014450	2105471	2025755	3025028	3046119	3073827
3rd	2020697	981999	997873	1071742	2015523	2104289	2026575	3024941	3039667	3075397
4th	2021768	981735	998424	1072459	2014666	2104476	2026777	3026257	3040631	3075694
5th	2021013	981091	998208	1072281	2015578		2027615	3025960	3039657	3075297
Average	2021463	982893.8	997811	1072073	2014557	2105180	2026626	3025106	3043544	3075054
RSD	0.03	0.26	0.10	0.03	0.06	0.05	0.03	0.04	0.17	0.03
Recovery		97.25	98.72	101.99	99.66	100.14	100.26	101.11	101.73	102.78
	Avg	99.32			100.02			101.88		
	RSD	2.43			0.32			0.84		
	Avg All	100.40								
	RSD All	1.72								

Table 6: Percentage recoveries of spiked hydrochlorothiazide

Accuracy Hydrochlorothiazide										
		50%			100%			150%		
	STD	T1	T2	T3	T1	T2	T3	T1	T2	T3
Wt	50.1	6.4	6.4	6.3	12.5	12.4	12.5	16.9	16.8	16.8
1st	1438426	759316	754643		1428912	1449801	1458898	1917704	1914225	
2nd	1436746	758938	757972	744878	1433293	1455258	1455714	1914930	1898582	1927926
3rd	1438774	756890	760831	740698	1433540	1450728	1454491	1920177	1900124	1930816
4th	1436020	757561	760932	741860	1429504	1448719	1452722	1912824	1898745	1930648
5th	1436935	757455	760895	745494	1431330		1456642	1915914	1900707	1929942
Avg	1437380	758032	759054.6	743232.5	1431316	1451127	1455693	1916310	1902477	1929833
RSD	0.08	0.14	0.36	0.31	0.15	0.20	0.16	0.15	0.35	0.07
Recovery		103.21	103.35	102.80	99.78	101.97	101.48	98.81	98.68	100.10
	Average	103.12			101.08			99.19		
	RSD	0.28			1.15			0.78		
	Average	101.13								
	RSD	1.82								

Specificity of the Method

It is noteworthy to mention that preliminary tests were performed whereby the specificity of the method was firstly determined against placebo. It was the found that there were no interferences between the drug and the excepients of the claimed placebo. Secondly the specificity of the method toward the drug was approved via the non-existence of interferences between the peaks of the drug and the degradation products resulting from exposure to forced stress conditions of acidic, alkaline, photolytic and oxidative conditions. In this context, it is important to mention that 24% and 8% of the drug was degraded during oxidative and alkaline stress conditions; while only traces of peaks were observed during exposure of the drug to photolytic and acidic conditions. In conclusion, no interferences were observed between the peaks of the drug and those of the degradation products.

CONCLUSION

A new analytical method has been developed to be routinely applied to simultaneous determination of amlodipine besylate and hydrochlorothiazide in pharmaceutical dosage form. In this study, stability of amlodipine besylate, hydrochlorothiazide in present dosage form was established through employment of ICH recommended stress conditions. The developed procedure has been evaluated over the specificity, linearity, accuracy, precision and robustness in order to ascertain the stability of the analytical method. It has been proved that it was specific, linear, precise, accurate and robust and stability indicating. Hence, the method is recommended for routine quality control analysis and also for stability sample analysis.

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