Development and Validation of a Reversed-phase HPLC Method for Simultaneous Determination of Aspirin, Atorvastatin Calcium and Clopidogrel Bisulphate in Capsules

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A simple, accurate, rapid and precise isocratic reversed-phase high-performance liquid chromatographic method has been developed and validated for simultaneous determination of aspirin, atorvastatin calcium and clopidogrel bisulphate in capsules. The chromatographic separation was carried out on an Inertsil ODS analytical column (150×4.6 mm; 5 µm) with a mixture of acetonitrile:phosphate buffer pH 3.0 adjusted with *o*-phosphoric acid (50:50, v/v) as mobile phase; at a flow rate of 1.2 ml/min. UV detection was performed at 235 nm. The retention times were 1.89, 6.6 and 19.8 min. for aspirin, atorvastatin calcium and clopidogrel bisulphate, respectively. Calibration plots were linear (r^2>0.998) over the concentration range 5-30 µg/ml for atorvastatin calcium and 30-105 µg/ml for aspirin and clopidogrel bisulphate. The method was validated for accuracy, precision, specificity, linearity, and sensitivity. The proposed method was successfully used for quantitative analysis of capsules. No interference from any component of pharmaceutical dosage form was observed. Validation studies revealed that method is specific, rapid, reliable, and reproducible. The high recovery and low relative standard deviation confirm the suitability of the method for routine determination of aspirin, atorvastatin calcium and clopidogrel bisulphate in bulk drug and capsule dosage form.

Key words: Aspirin, atorvastatin calcium, clopidogrel bisulphate, HPLC

Aspirin (ASP) (fig.1a) is a well-known antithrombotic, antipyretic, analgesic agent. Chemically 2-(acetyloxy)benzoic acid, it is official in USP-NF[1], BP[2] and IP[3]. It is an antiplatelet agent approved by the Food and Drug Administration, USA, for use in secondary prevention of heart attacks and stroke[4,5]. Besides it is mainly used as an analgesic, antipyretic, antiinflammatory and antithrombic agent. Atorvastatin calcium (ATO) (3*R*,5*R*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid, calcium salt, (2:1) trihydrate) (fig.1b), a synthetic lipid-lowering agent is official in IP[3]. It is a selective competitive inhibitor of the enzyme HMG-CoA reductase, which catalyses the conversion of HMG-CoA to mevalonate, an important rate limiting step in cholesterol biosynthesis[6].

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Clopidogrel bisulphate (CLO) (+)-(*S*)-methyl2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-*c*] pyridin-5(4*H*)-yl)acetate (fig.1c), sulfate is a new thienopyridine derivative. It is an antiplatelet agent, which selectively inhibits the binding of adenosine diphosphate (ADP) to its platelet receptor and blocks the subsequent ADP-mediated activation of the glycoprotein GPIIb/IIIa complex, thereby inhibiting platelet aggregation[7]. It is official in USP-NF[8]. It has been shown to prevent ischemic stroke, myocardial infraction and vascular disease and has demonstrated its clinical efficacy superior to that of aspirin. Thus, clopidogrel is indicated for the patients with atherosclerosis documented by recent stroke, recent myocardial infraction or cardiovascular disease[9]. The ternary combination of ASP, ATO and CLO is used for atherosclerotic patients suffering from various heart diseases.

Literature survey reveals many reported methods for the analysis of ASP by high-performance

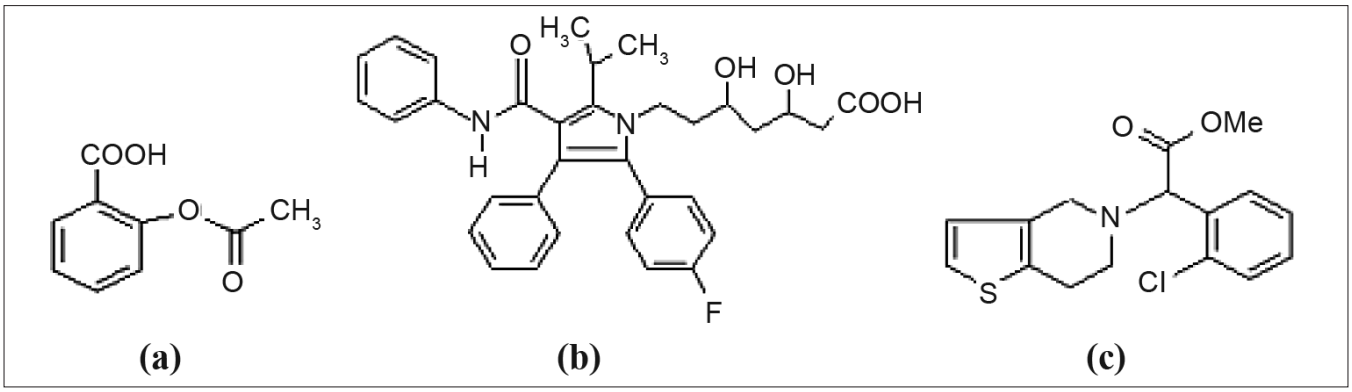

Fig. 1: Chemical structures of the analytes.
Structures of (a) aspirin, (b) atorvastatin calcium and (c) clopidogrel bisulphate (c)

liquid chromatography (HPLC)[10-12] and high-performance thin-layer chromatography (HPTLC)[13]. Chromatographic methods have been reported for determination of ATO, in combination with other drugs, in bulk and pharmaceutical dosage forms[14-18]. Estimation of CLO by HPLC[19-21] and HPTLC, either individually or in combination with other drugs is reported[22,23].

To the best of our knowledge hitherto there is no analytical method reported for simultaneous determination of ternary mixture containing ASP, ATO and CLO. Therefore, an attempt has been made to develop a simple, accurate, rapid and reproducible reverse phase HPLC method for simultaneous determination of ASP, ATO and CLO in capsule dosage form and validate it, in accordance with ICH guidelines[24].

MATERIALS AND METHODS

Pharmaceutical grade of ASP, ATO and CLO were kindly supplied as gift samples by Torrent Pharmaceuticals, Gujarat, India, certified to contain >99% (w/w) on dried basis. Commercially available Ecosprin Gold-10 (Tristar Formulation Pvt. Ltd., Pondicherry, India) and Deplatt-CV (Surien Pharmaceuticals, Pondicherry, India) capsules claimed to contain 75 mg aspirin; 10 mg atorvastatin calcium and 75 mg clopidogrel bisulphate have been utilized in the present work. All chemicals and reagents used were of HPLC grade and were purchased from Merck Chemicals, India.

Chromatographic system and conditions:
The HPLC system consisted of a Perkin Elmer (USA) Series 200 instrument equipped with a binary pump, rheodyne injector with 20 µl capacity loop and UV detector. The software used was Total Chrom Navigator version® 6.3. Separation was achieved on a reverse phase Inertsil® ODS analytical column (150×4.6 mm; 5 µm). The mobile phase consisted of acetonitrile and 10 mmol/l potassium phosphate buffer adjusted to pH 3.0 with *o*-phosphoric acid in the ratio 50:50 (v/v). Before analysis the mobile phase was filtered through a 0.2 µm membrane and degassed by ultrasonification. The flow rate was 1.2 ml/min. Detection was monitored at 235 nm and injection volume was 20 µl. All the experiments were performed at ambient temperature.

Standard solutions and calibration graphs for chromatographic measurement:
Stock standard solutions were prepared by dissolving separately 100 mg of ASP, ATO and CLO in 100 ml methanol (1000 µg/ml). The standard calibration solutions were prepared by appropriate dilution of the stock solution with methanol to reach a concentration range of 30-105 µg/ml for ASP and CLO and 5-30 µg/ml for ATO. Triplicate 20 µl injections were made for each concentration and chromatographed under the optimized conditions described above. The peak area were plotted against the corresponding concentrations to obtain the calibration graphs.

Sample preparation:
Twenty capsule contents were accurately weighed, their mean weight was determined and they were mixed and finely powdered. A portion equivalent to about one capsule was accurately weighed and transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 30 min and diluted to 100 ml with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min. Supernatant was taken and after suitable dilution the sample solution was then filtered using 0.45 µ filter (Millipore,

Milford, MA). The original stock solution was further diluted to get sample solution of drug concentration of 75 µg/ml ASP, 10 µg/ml ATO and 75 µg/ml CLO. A 20 µl volume of sample solution was injected into HPLC, six times. The peak areas for the drugs were measured at 235 nm and amounts of ASP, ATO and CLO were determined using the related linear regression equations.

Method validation:

The developed method was validated according to the ICH guidelines[24]. The system suitability was evaluated by six replicate analyses of ASP, ATO and CLO mixture at a concentration of 75 µg/ml ASP, 10 µg/ml ATO and 75 µg/ml CLO. The acceptance criteria were a R.S.D. of peak areas and retention times less than 2%, Theoretical plate numbers (N) at least 2500 for each peak and tailing factors (T) less than 1% for ASP, ATO and CLO.

Standard calibration curves were prepared in the mobile phase with six concentrations ranging from 30-105 µg/ml for ASP and CLO and 5-30 µg/ml for ATO in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs. To study the reliability and suitability of the developed method, recovery experiments were carried out at three levels 80, 100 and 120%. Known concentrations of commercial capsules were spiked with known amounts of ASP, ATO and CLO. At each level of the amount six determinations were performed and the results obtained were compared with expected results. Recovery for pharmaceutical formulations should be within the range 100±5%. The percent R.S.D. of individual measurements was also determined. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) for 3 consecutive days. Three different concentrations of ASP, ATO and CLO were analyzed in six independent series in the same day (intra-day precision) and 3 consecutive days (inter-day precision). Every sample was injected in triplicate. The repeatability of sample application and measurement of peak area for active compounds were expressed in terms of percent RSD.

All chromatograms were examined to determine if compounds of interest co-eluted with each other or with any additional excipients peaks. Marketed formulations were analyzed to determine the specificity of the optimized method in the presence of common capsule excipients. Limit of detection (LOD) and limit of quantitation (LOQ) were estimated from the signal-to-noise ratio. LOD and LOQ were calculated using $3.3\sigma/s$ and $10\sigma/s$ formulae, respectively, where, σ is the standard deviation of the peak areas and s is the slope of the corresponding calibration curve. To evaluate robustness of HPLC method a few parameters were deliberately varied. The parameters included variation of flow rate, percentage of buffer in the mobile phase, and pH of mobile phase.

RESULTS AND DISCUSSION

During the optimization of HPLC method, two columns (HI-Q Sil C18 5µm; 250 mm × 4.6 mm and Inertsil ODS C18 5 µm; 150×4.6 mm), two organic solvents (acetonitrile and methanol), two buffers (acetate and phosphate) at two different pH values (3 and 4) were tested. Initially methanol:water, acetonitrile:water, acetonitrile:acetate buffer, methanol:acetate buffer were tried in different ratios at pH 3 and 4. ASP and ATO eluted with the tried mobile phases, but CLO was retained. Then, with acetonitrile: phosphate buffer all the three drugs eluted, but the analysis time was more than 30 min. In order to decrease the analysis time, column length was reduced from 250 to 150 mm. The mobile phase conditions were optimized so the peak from the first-eluting compound did not interfere with those from the solvent, excipients. Other criteria, *viz.* time required for analysis, appropriate k range ($1<k<10$) for eluted peaks, assay sensitivity, solvent noise were also considered. Finally a mobile phase consisting of a mixture of acetonitrile: phosphate buffer pH 3.0 adjusted with *o*-phosphoric acid in ratio 50:50 (v/v), was selected as mobile phase to achieve maximum separation and sensitivity. Flow rates between 0.8 to 1.4 ml/min were studied. A flow rate of 1.2 ml/min gave an optimal signal to noise ratio with a reasonable separation time. Using a reversed phase C18 column, the retention times for ASP, ATO and CLO were observed to be 1.89, 6.6, 19.8 min, respectively. Total time of analysis was less than 20 min. The chromatogram at 235 nm showed a complete resolution of all peaks (fig. 2).

Validity of the analytical procedure as well as the resolution between different peaks of interest is

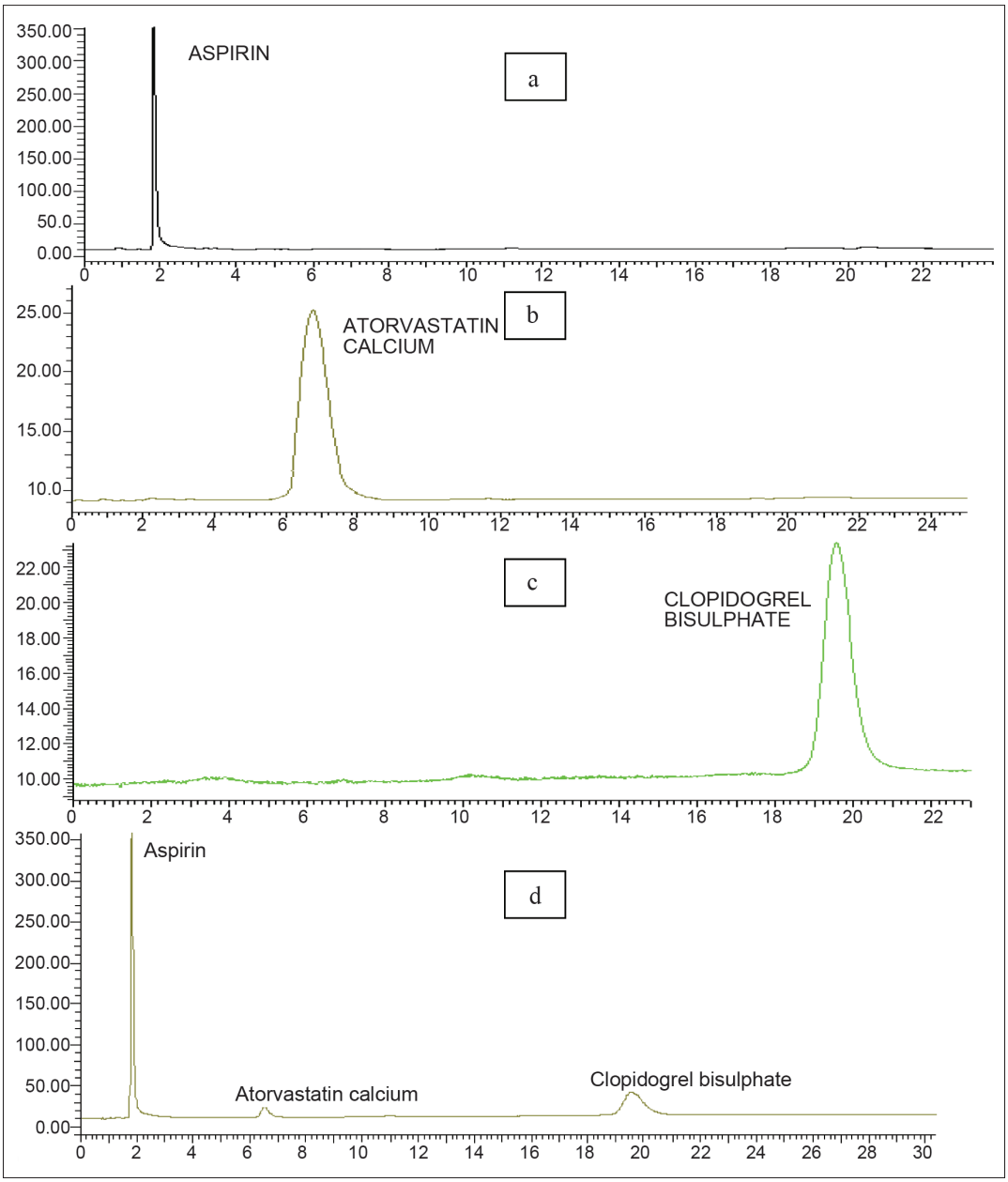

Fig. 2: Representative chromatograms of standard solutions
(a) Standard solution of ASP (75 µg/ml); (b) standard solution of ATO (10 µg/ml); (c) standard solution of CLO (75 µg/ml) and (d) a standard solution containing 75 µg/ml ASP, 10 µg/ml ATO, 75 µg/ml CLO.

ensured by the system suitability test. All critical parameters tested met the acceptance criteria on all days. As shown in the chromatogram, all three analytes are eluted by forming symmetrical single peaks well separated from the solvent front (fig. 2).

Excellent linearity was obtained for all the three drugs in the range of 30-105 µg/ml for ASP and ATO and 5-30 µg/ml CLO. The correlation coefficients (r^2) were found to be greater than 0.999 (n=6) in all instances. The results of calibration studies are summarized in Table 1. The proposed method afforded high recoveries for ASP, ATO and CLO capsules. Results obtained from recovery studies presented in Table 2, indicate that this assay procedure can be used for routine quality control

TABLE 1: LINEARITY PARAMETERS FOR THE SIMULTANEOUS ESTIMATION OF ASP, ATO AND CLO (*N=6*)

Parameters	ASP	ATO	CLO
Linearity range µg/ml	30-105	5-30	30-105
r^2±SD	0.9997±0.6	0.9983±0.26	0.9984±0.48
Slope±SD	0.32±0.22	0.34±0.74	0.24±0.82
Intercept±SD	22.14±0.18	-6.63±0.56	-24.42±0.88

TABLE 2: RESULTS OF ACCURACY STUDIES OF ASP, ATO AND CLO

Label claim (mg/capsule)	Amount added (%)	Total amount added (mg)	Amount recovered (mg)	% Recovery±SD*	% RSD
ASP (75)	80	135	134.47	99.61±0.55	0.56
	100	150	148.22	98.81±0.61	0.28
	120	165	165.12	100.35±0.83	0.53
ATO (10)	80	18	17.96	98.28±0.49	0.69
	100	20	20.12	100.70±0.26	0.80
	120	22	22.02	100.21±0.27	0.44
CLO (75)	80	135	135.24	100.59±0.25	0.25
	100	150	149.42	99.41±0.11	0.21
	120	165	165.20	100.52±0.33	0.23

*n=6, SD: Standard deviation, % RSD: Relative standard deviation

TABLE 3: RESULTS OF INTRA-DAY PRECISION AND INTER-DAY PRECISION FOR SIMULTANEOUS DETERMINATION OF ASP, ATO AND CLO STANDARDS

Compound	Intra-day precision (n=6)		Inter-day precision (n=6)	
	% RSD	SE	% RSD	SE
ASP	1.51	0.76	1.03	0.55
ATO	1.06	0.39	1.24	0.68
CLO	1.12	0.59	0.92	0.85

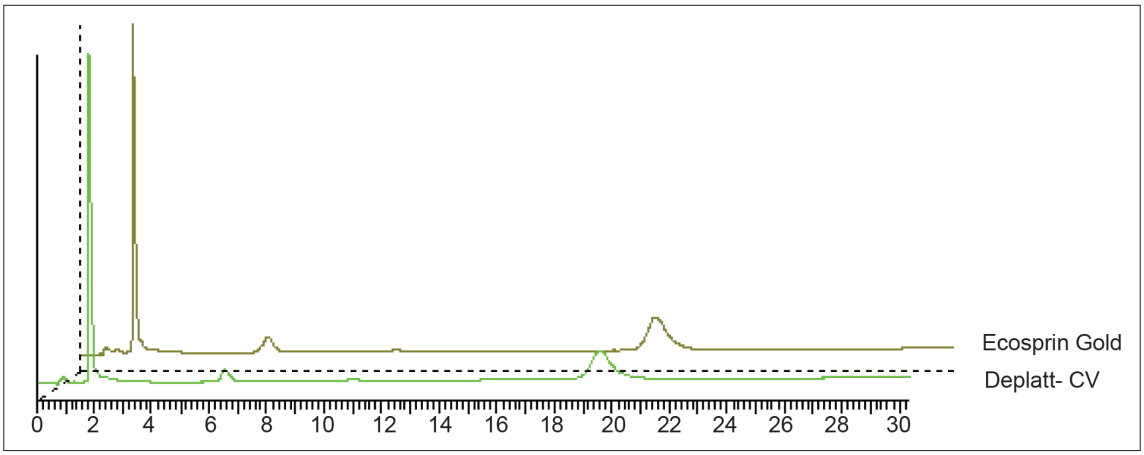

Fig. 3: : Representative chromatogram obtained for marketic formulations.
Representative chromatogram obtained for Ecosprin Gold-10 and Deplatt CV capsule formulations

analysis of this ternary mixture in capsules. Precision of the analytical method was found to be reliable based on % RSD (<2%) corresponding to the peak areas and retention times. As can be seen in Table 3, the % RSD values were less than 2, for intra-day and inter-day precision. Hence, the method was found to be precise for all the three drugs.

The chromatograms were checked for the appearance of any extra peaks. It was observed that single peak for ASP (R_t±SD, 1.89±0.01), ATO (R_t±SD, 6.6±0.01) and CLO (R_t±SD, 19.8±0.01) were obtained under optimized conditions, showing no interference from common capsule excipients and impurities. Also the peak areas were compared with the standard and % purity calculated was found to be within the limits. These results demonstrate the specificity of the method (fig. 3).

LOD and LOQ were found to be 1.8 µg/ml and 5.5 µg/ml for ASP, 0.0959 µg/ml and 0.290 µg/ml for ATO and 0.66 µg/ml and 1.89 µg/ml for CLO. In all deliberately varied conditions, the SD of retention times of ASP, ATO and CLO were found to be well within the acceptable limit. The tailing factor for all the three peaks was found to be <1.5 (Table 4). The validated method was used in the analysis of marketed conventional capsules Ecosprin Gold-10 and Deplatt-CV with a label claim: 75 mg ASP, 10 mg ATO and 75 mg CLO per capsule. Representative

TABLE 4: RESULTS OF ROBUSTNESS STUDY

Factor[b]	Level	ASP		ATO		CLO	
		T_r[c]	T.F	T_r[c]	T.F	T_r[c]	T.F[d]
A: Flow rate							
1.0	-1	1.92	1.1	6.7	0.8	19.86	1.22
1.2	0	1.89	0.8	6.6	0.6	19.8	1.3
1.3	1	1.87	1.4	6.56	1.1	19.72	1.14
Mean±SD (n=6)		1.893±0.025	1.1±0.05	6.62±0.072	0.83±0.035	19.79±0.070	1.22±0.10
B: Percentage of buffer in mobile phase(v/v)							
49	-1	1.86	1.2	6.5	0.94	19.7	1.24
50	0	1.89	0.78	6.6	0.9	19.8	1.1
51	1	1.90	1.3	6.66	0.86	19.88	0.96
Mean±SD(n=6)		1.88±0.020	1.09±0.016	6.586±0.080	0.9±0.08	19.81±0.056	1.1±0.21
C: pH of mobile phase							
2.9	-1	1.86	1.3	6.52	1.06	19.68	0.5
3.0	0	1.89	1.1	6.6	0.58	19.8	1.04
3.1	1	1.94	1.4	6.7	1.3	20.2	0.88
Mean±S.D.(n=6)		1.896±0.040	1.26±0.065	6.63±0.133	0.98±0.050	19.89±0.27	0.78±0.082

[a]concentration used was 20 µg/ml, [b]Three factors were slightly changed at three different levels (-1, 0, 1), [c]Retention time, [d]Tailing factor

TABLE 5: RESULTS OF ASSAY IN COMMERCIAL SAMPLES

Compound	Label claim (mg)	Ecosprin Gold-10 capsules		Deplatt-CV capsules	
		Amount found (mg)	% Drug content*	Amount found (mg)	% Drug content*
ASP	75	74.15	98.87±0.24	75.15	100.3±0.54
ATO	10	10.12	101.20±0.074	9.98	99.80±0.22
CLO	75	73.90	98.53±0.43	74.5	99.36±0.16

*n=6

chromatogram is shown in (fig. 3). The results for the drugs assay show a good agreement with the label claims (Table 5).

The developed HPLC method is simple, specific, accurate and precise for the simultaneous determination of ASP, ATO and CLO from capsules. The developed method provides good resolution between ASP, ATO and CLO. It was successfully validated in terms of system suitability, linearity, range, precision, accuracy, specificity, LOD, LOQ and robustness in accordance with ICH guidelines. Thus, the described method is suitable for routine analysis and quality control of pharmaceutical preparations containing these drugs either as such or in combination.

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