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Quantitation and Validation of Atorvastatin using HPLC-UV

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ABSTRACT – A reversed phase HPLC analysis of atorvastatin (AS) standard solution was performed using diclofenac (DF) as internal standard. Column oven temperature, flow rate and the composition of the mobile phase were varied in order to determine a practical system setup using a C18 column and UV detector. Two C18 columns of different length were compared regarding their influence on the AS peak shape. Based on these preliminary experiments a validation study was performed utilizing a C18 column at 62°C with a mobile phase consisting of sodium phosphate buffer (0.05 M, pH 4.0), methanol and acetonitrile (40 : 50 : 10, v/v/v). The detection limit for AS was 0.1 μg/ml and inter- and intra-day calibration curves were linear over a concentration range of 0.2 - 50 μg/ml. Accuracy and precision were satisfactory in the AS concentration range of 0.5 - 50 μg/ml.

Key words – Atorvastatin, HPLC, UV, Validation

Statins are agents that are used in the pharmaceutical industry as lipid-lowering-drugs (LLDs). They exhibit activity by inhibiting HMG-CoA reductase, an enzyme involved in an early, rate-limiting step during cholesterol synthesis. They are used for the treatment of hyperlipidemia - the accumulation of lipids in the blood. Hyperlipidemia is a syndrome connected to deadly diseases such as coronary heart disease or strokes because the higher level of cholesterol in the blood might lead to its deposition in the arterial cell wall or in the cell wall of the vessels leading to the brain. Statins have been found to effectively lower the blood cholesterol level and form an effective hyperlipidemia therapy when combined with dietary and lifestyle changes.[1)]

Atorvastatin (AS, Figure 1) [R-(R*, R*)]-2-(4-fluorophenyl)-beta,delta-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid - CAS 134523 -00-5, is one of the statins which also reduces the concentration of triglycerides in the blood[2)] and was FDA approved in December of 1996. For medical studies and routine check-ups the determination of statin concentrations in a patient's blood sample is necessary. Several studies have been published describing liquid chromatography with a tandem mass detection (LC-TMS) for these purposes.[3,4,5)]

However, the LC-TMS analysis requires an expensive instrumentation which is not readily available. In the process of developing novel formulations for AS, an alternative method to the LC-TMS that is cost effective, easy to handle yet yielding high sensitivity and reproducibility needs to be sought for. Moreover, statins including AS are generally difficult to analyse due to their strong adherence to the HPLC columns, causing an increase in column pressure.

`Recently, studies have been published in which a reversed-phase high-pressure liquid chromatography with an UV/Vis detection (HPLC-UV) was used for the detection of atorvastatin in human plasma samples,[6,7)] beagle dog plasma samples [8)] and bulk tablet samples.[2,9)] One study reported on the simultaneous detection of several statins.[10)] Pharmacokinetic studies indicated that the AS concentration in human serum after oral adminstration of 40 mg AS - a mid-range dose on the market - did not exceed 50 ng/ml (0.05 μg/ml).[6)] This might be no problem with a mass detection but might become critical in terms of sensitivity if working with an UV detection. The aim of this study, therefore, was to validate the analysis of AS using HPLC-UV with diclofenac (DF) as internal standard.

Experimental

Materials

Atorvastatin calcium salt (AS) was purchased from Beijing HuaFeng United Technology Co., Ltd, Beijing, China. The internal standard (diclofenac sodium salt, DF), sodium phosphate monobasic and sodium phosphate dibasic heptahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), methanol (MeOH) and water were of

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HPLC grade and obtained from J.T. Baker (NJ, USA). o-Phosphoric acid was purchased from PFP Matsuonen Chemicals LTD. (Osaka, Japan). All reagents were stored at ambient temperature except AS which was stored at − 24°C.

Preparation of standard solutions and buffer

An 800 μg/ml stock solution of AS (80 mg AS in 100 ml MeOH) was diluted to concentrations in the desired range. This stock was used for the experiments with the Merck column. Another AS stock with 400 μg/ml in MeOH was prepared for the intra- and inter-day validation using a Shiseido column. A 400 μg/ml stock solution of DF in MeOH was prepared and diluted to a working solution of 8 μg/ml. Sodium phosphate buffer (0.05 M) was prepared in water with sodium phosphate monobasic and sodium phosphate dibasic heptahydrate. The pH was adjusted to 4.0 with σ-phosphoric acid. The buffer and the standard solutions were stored at 2°C.

Sample preparation

The AS stock was diluted to samples with concentrations twice as high as the desired. To obtain the final samples for analysis, one ml of the respective AS dilution (AS concentration halved) was combined with one ml of the DF working solution (final DF concentration of 4 μg/ml). All samples were stored at 2°C.

Instrumentation and chromatographic conditions

The HPLC analysis was performed using a Waters 2690 separations module (Waters, MA, USA) to which a C18 column was connected. Throughout this study two different C18 columns were used. A LiChroCART® 125-4 LiChrospher® 100 RP-18 (5 μm) column (Merck, Darmstadt, Germany) with a LiChroCART® 4-4 LiChrospher® 100 RP-18 (5 μm) guard column (Merck, Darmstadt, Germany) were utilized for the initial experiments to determine the system parameters. The final validation was done without a guard column on a CAPCELL PAK C18 MG S5 column (Shiseido, Osaka, Japan). The column temperature was controlled by a Waters column heater module. For UV detection a Waters 2487 Dual Absorbance Detector operated at a wavelength of 247 nm was used. The data was processed by an AutoChro 2000® software. Sample injections were performed manually with a 25 μl syringe (Hamilton, NE, USA). The sample loop had a volume of 20 μl. MeOH, water and mobile phase were filtered through a 0.2 μm pore-sized filter (Sartorius, Goettingen, Germany) with an all glass apparatus connected to a vacuum pump and degassed with a BRANSONIC® 8510 tabletop ultrasonic cleaner (Branson, CT, USA). The pH of the sodium phosphate buffer was adjusted using a Corning pH meter 440 (Corning, NY, USA). AS, DF and the salts for the sodium phosphate buffer were weighed out on a Sartorius CP225D semi micro balance (Sartorius, Goettingen, Germany). For validation experiments, the column temperature was set to 62°C and mobile phase variant F (Table I) was pumped at a flow rate of 1.5 ml/min.

Determination of chromatographic conditions

The influence of the polarity of the mobile phase on the quality of chromatographic separation was checked by changing the proportions of buffer and solvent at constant column temperature (62°C) and constant flow rate (1.5 ml/min). Here several different variants were tested (Table I), whereas firstly the polarity had to be increased (variants B-D) to obtain a successful separation of the analyte peak from the standard peak. By adding certain amounts of ACN to the mobile phase, the effect on the AS peak shape was examined with variants E-F. We also did injections at different flow rates in the range of 1.0 − 1.8 ml/min (leaving column temperature and mobile phase composition unchanged) in order to determine the influence of the flow rate on the peak shape and resolution of AS and DF. Finally the influence of the column temperature on the system parameters at a constant flow rate and an unchanged mobile phase composition was checked as well. Measurements were performed at four different column temperatures: 32, 42, 62 and 72°C. The respective resulting system parameters were compared and evaluated. With all parameter variations that were performed it was aimed at obtaining a successful separation of the AS and DF peaks, optimal peak shapes, as well as a system pressure below 2000 PSI.

Method validation

The intra-day variations were obtained by measuring the samples in triplicates, respectively. Inter-day variation data was assessed by doing a repeated analysis in 4 analytical runs on different days. A calibration curve for the AS concentration range of 0.2 - 50 μg/ml was plotted and its sensitivity, linearity and accuracy were determined. The lower limit of detection (LOD) was defined as the lowest concentration of AS quantified with a CV of approximately 20%. Calibration curves were obtained by a linear least-square regression plot of the peak-area ratios (AS/IS) versus the respective AS concentrations with Microsoft Excel.

Results and Discussion

Mobile phase composition

Using the mobile phase variant A - composed of 0.05 M

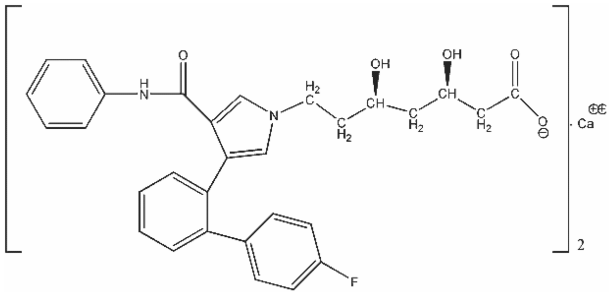

Figure 1–Chemical structure of atorvastatin calcium salt.

Table I–*Composition of Mobile Phase Variants*

Variant	MeOH [v%]	SPB [v%]	ACN [v%]
A	67	33	-
B	60	40	-
C	55	45	-
D	50	50	-
E	55	40	5
F	50	40	10
G	48	42	10
H	45	45	10

MeOH, Methanol
SPB, Sodium phosphate buffer (0.05 M, pH 4.0)
ACN, Acetonitrile

Table II–*Retention Times and Resolutions for Different Mobile Phase Compositions.*

Mobile phase variant	FR [ml/min]	tr_{DF} [min]	tr_{AS} [min]	Rs
A	1	2.01	2.39	0.27
B	1	3.31	5.26	1.70
C	1	5.08	10.06	6.23
E	1.5	3.50	4.86	1.44
F	1.5	3.10	4.17	1.07

FR, Flow rate
tr_x, Retention time of X
Rs, Resolution, $Rs=2(tr_{AS}-tr_{DF})/(W_{DF}+W_{AS})$

sodium phosphate buffer (pH 4.0) and MeOH (33/67, v:v) - and the C18 Merck column at 62°C did not lead to a satisfying separation of the AS and the DF peak. Consequently an attempt was made to obtain a good separation of the AS and DF peaks by changing the polarity of the mobile phase. Several variants were tried (Table I). With variant B, a successful separation of the analyte and the internal standard peak but not a satisfying AS peak shape was achieved. Using variant D the AS peak shape was slightly improved. However the retention time for AS was about 22 min, which needed to be shortened. An improvement of the AS peak shape could be achieved by adding certain amounts of ACN instead of MeOH to the mobile phase (variants E-H). Other variants were also tested (C, G, H) but did not lead to results that were significantly different. Finally variant F was chosen to be used as mobile phase for further analysis because its resulting system pressure was 100 PSI lower than the one obtained with variant E. The retention times and resolutions for different mobile phase compositions for AS and DF can be seen in Table II.

Flow rate

The influence of the flow rate on the system parameters, mainly on the shape of the AS peak, was studied. A column temperature of 62°C with mobile phase variant B and a sample with an AS concentration of 4 μg/ml was used. Starting at 1.0 ml/min the flow rate was increased until the desired resolution and peak shape were obtained. The system pressure always had to be watched carefully. The following flow rates were tested: 1.0, 1.5, 1.6 and 1.8 ml/min. All in all this experiment led to the following conclusions: The higher the chosen flow rate the higher was the resulting system pressure (Table III). In terms of the AS peak shape good progress was made by increasing the flow rate from 1.0 to 1.5 ml/min. While changing it from 1.5 to 1.6 and 1.8 ml/min could only yield minor improvements. The most symmetrical AS peak was obtained at a flow rate of 1.8 ml/min. Yet it was decided to work with a flow rate of 1.5 ml/min in regard to a lower system pressure and a better resolution.

Column temperature

The influence of the column temperature on the system's parameters (p, to, tr), the quality of the separation (K, α) and the quality of the peak shape were observed. The column was heated up to four different temperatures (32, 42, 62 and 72°C) while the other parameters of the chromatographic setup remained constant (the same mobile phase, column, flow rate and sample were used). A sample with an AS concentration of 0.5 μg/ml and a mobile phase flow rate of 1.5 ml/min was

Table III–*System Pressure, Retention Times and Resolution at Different Flow Rates for Mobile Phase Variant B and a Column Temperature of 62°C.*

FR [ml/min]	tr_{DF} [min]	tr_{AS} [min]	Rs	p [psi]
1	3.31	5.26	1.70	950
1.5	2.53	4.02	1.32	1650
1.6	2.48	3.80	1.10	1750
1.8	2.22	3.49	1.21	1950

FR, Flow rate
tr_x, Retention time of X
Rs, Resolution, $Rs=2(tr_{AS}-tr_{DF})/(W_{DF}+W_{AS})$
p, System pressure

Table IV–*Effects of Column Temperature on Chromatographic Parameters at 32°C(A), 42°C(B), 62°C(C) and 72°C(D)*

A

run	to	tr_{IS}	tr_{AS}	K_{IS}	K_{AS}	α
1st	0.7000	4.8417	6.4167	5.9167	8.1667	1.3803
2nd	0.7083	4.8217	6.4200	5.8074	8.0640	1.3886
3rd	0.7083	4.8167	6.3850	5.8004	8.0145	1.3817
			mean	5.8415	8.0817	1.3835
			SD	0.0652	0.0776	0.0044

B

run	to	tr_{IS}	tr_{AS}	K_{IS}	K_{AS}	α
1st	0.7250	4.0400	5.4033	4.5724	6.4528	1.4113
2nd	0.7283	4.0633	5.4467	4.5792	6.4786	1.4148
3rd	0.7217	4.0717	5.4583	4.6418	6.5631	1.4139
			mean	4.5978	6.4982	1.4133
			SD	0.0383	0.0577	0.0019

C

run	to	tr_{IS}	tr_{AS}	K_{IS}	K_{AS}	α
1st	0.7200	3.0767	4.1317	3.2732	4.7385	1.4477
2nd	0.7167	3.0817	4.1217	3.2998	4.7509	1.4397
3rd	0.7138	3.0733	4.1267	3.3055	4.7813	1.4465
			mean	3.2929	4.7569	1.4446
			SD	0.0173	0.0220	0.0043

D

run	to	tr_{IS}	tr_{AS}	K_{IS}	K_{AS}	α
1st	0.7150	2.6183	3.4617	2.6620	3.8415	1.4431
2nd	0.7133	2.6100	3.4383	2.6590	3.8203	1.4367
3rd	0.7150	2.6083	3.4633	2.6480	3.8438	1.4516
			mean	2.6563	3.8352	1.4438
			SD	0.0074	0.0130	0.0075

K, Capacity Factor $K=(tr-to)/to$
to, Retention Time of Solvent [min]
tr_x, Retention Time of Compound x [min]
α, Separation Factor / Selectivity $\alpha=K_{AS}/K_{IS}$

used. The sample was injected in triplicates for each column temperature. The results for the observed parameters can be found in Table IV. Retention times were listed up and the capacity and separation factors were calculated according to the respective formulas also given in that table. The retention times and the system pressure of the internal standard and the analyte decreased as temperature increased, whereas the separation factor of the two peaks was steadily rising with rising temperature. In Figure 2, the capacity factors of AS and DF were plotted versus the respective temperature and a polynomial regression for both plots was performed. This type of regression yielded a very high coefficient of determination (R^2) value for both substances and suggests a polynomial temperature dependence for the capacity factors. The AS and DF peak shapes (not shown) seemed not to be influenced by the different column temperatures. Moreover, the problem of high column pressure due to the "sticky" nature of AS was solved by the increase in temperature. In respect to the system pressure and the retention times of DF and AS, the working temperature of 62°C was chosen for method validation studies.

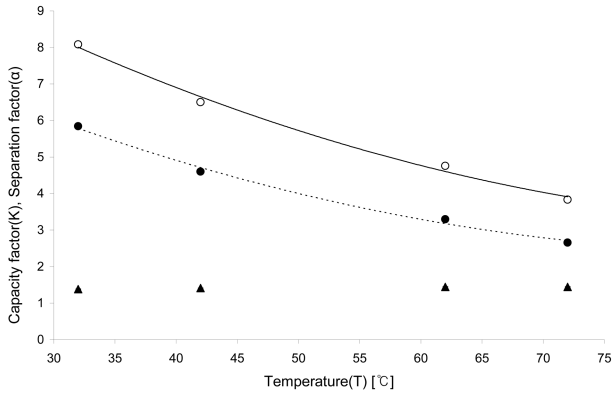

Figure 2–Plots of the capacity factors of the internal standard (DF) K_{IS} (○), that of AS K_{AS} (●) and their separation factor $\alpha=K_{AS}/K_{IS}$ (▲) vs. the respective column temperature. Polynomial regressions were shown for DF (- $y=0.0011x^2-0.2171x+13.822$, $R^2=0.9945$) and AS (····· $y=0.001x^2-0.182x+10.572$, $R^2=0.9945$).

Column length

Two C18 columns with different geometries (see instrumentation) were utilized to determine the influence of the column length on the AS peak shape. This was done by injecting the same analyte sample while keeping all other system parameters constant (The same mobile phase, flow rate and column temperature were used). The 25 cm column yielded a sharper AS peak (Figure 3) than the 12.5 cm (Figure 4) column while retention times were shorter and the system pressure was higher for the latter one. Consequently the 25 cm column was chosen for further validation studies.

Obtained chromatogram and retention times

With the chromatographic conditions used for the validation (Shiseido column at 62°C, mobile phase variant F and a flow rate of 1.5 ml/min) DF and AS were eluted at retention times of 5.7 and 7.8 mins, respectively. A representative chromato-

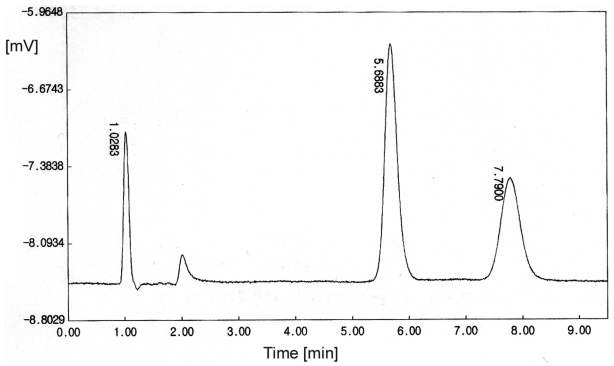

Figure 3–HPLC chromatogram of AS 1 μg/ml sample (spiked with 4 μg/ml of DF) obtained using the Shiseido column. Mobile phase was 0.05 M sodium phosphate buffer (pH 4.0)/MeOH/ACN (40/50/10, v/v/v). Column temperature was 62°C and flow rate, 1.5 ml/min.

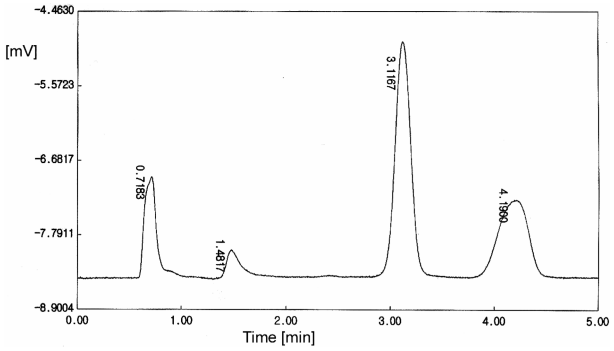

Figure 4–HPLC chromatogram of AS 1 μg/ml sample (spiked with 4 μg/ml of DF) obtained using the Merck column. Mobile phase was 0.05 M sodium phosphate buffer (pH 4.0)/MeOH/ACN (40/50/10, v/v/v). Column temperature was 62°C and flow rate, 1.5 ml/min.

gram can be seen in Figure 3.

Sensitivity, linearity and calibration curves

The LOD was at approximately 0.1 μg/ml. Standard calibration curves were linear over the concentration range of 0.2-50 μg/ml. The correlation coefficients for the linear least-squares regression were equal to or better than 0.9989. The results for the inter-day reproducibility (n=4) can be seen in Figure 5.

Accuracy and precision

A summary of the accuracy and precision results for the assessed intra- and inter-day calibration curve in the AS concentration range of 0.2-50 μg/ml can be found in Table V. Except for the coefficient of variation (CV) values at 0.2 μg/ml, the CV values for the intra- and inter-day measurements were all less than 6.5%. The rather high CV values at 0.2 μg/ml indicate that this concentration is relatively close to the method's LOD. The accuracy of the method was 81.19-111.09 (intra-day) and 92.92-114.04 (inter-day). Lowest accuracy was

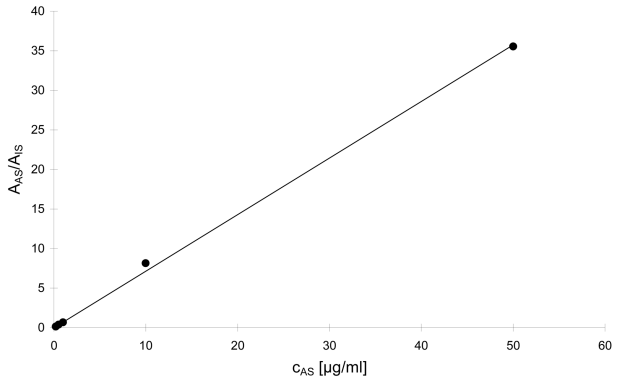

Figure 5–Linear range (0.2-50 μg/ml) of calibration curve (peak area ratio AS/IS vs. C_{AS}) which showed a correlation of y=0.7149 x (R^2=0.9989). This calibration curve was used for the inter-day validation data (n=4).

Table V–*Precision and Accuracy Results of the Validation Study for AS*

Known concentration [ppm]	Concentration found [mean ± SD]	Coefficient of variation [%]	Accuracy [%]	SD (Accuracy) [%]
Intra-day (n=3)				
0.2	0.162 ± 0.031	18.86	81.19	15.31
0.5	0.484 ± 0.031	6.41	96.89	6.21
1	0.937 ± 0.043	4.64	93.72	4.35
10	11.109 ± 0.084	0.76	111.09	0.84
50	49.783 ± 1.168	2.35	99.57	2.34
Inter-day (n=4)				
0.2	0.186 ± 0.031	16.58	92.92	15.41
0.5	0.528 ± 0.024	4.53	105.68	4.79
1	0.957 ± 0.054	5.62	95.74	5.38
10	11.404 ± 0.054	2.16	114.04	2.46
50	49.719 ± 2.582	5.19	99.44	5.16

obtained at 0.2 μg/ml and 10 μg/ml. The most accurate data could be obtained for an AS concentration of 50 μg/ml.

Conclusion

A relatively simple and cost effective analytical method for atorvastatin has been validated using an HPLC-UV system. Although the method is not as sensitive as that done with an LC-Mass, it would be sufficient for analysis of *in vitro* permeation or dissolution studies of atorvastatin.

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