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Validation of a shielded-hydrophobic-phase high-performance liquid chromatography method for the determination of residual methotrexate in recombinant protein biopharmaceuticals

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Abstract

A shielded-hydrophobic-phase (SHP) HPLC method for the determination of residual methotrexate in recombinant protein biopharmaceuticals was validated. The method requires no removal of protein or other prior sample ''clean-up'' and detects quantities of methotrexate as low as 2.5 ng in the presence of up to 25 mg/ml of protein. Methotrexate was fully resolved from a recombinant IgG1 monoclonal antibody and associated matrix components. Accuracy was demonstrated by measuring ''spiked'' recoveries at the limit of quantitation (found 90–120% recovery with R.S.D.s $\leq$10%). Other validation parameters studied included range, precision, ruggedness, robustness and stability of ''spiked'' samples. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: Shielded-hydrophobic-phase high-performance liquid chromatography; Stationary phases, LC; Validation; Pharmaceutical analysis; Methotrexate; Proteins

1. Introduction

Methotrexate, L-(+)-*N*-(4-{[(2,4-diamino-6-pteridinyl)methyl]-methylamino}-benzoyl)-glutamic acid, a potent inhibitor of the dihydrofolate reductase (DHFR) enzyme [1], is a component of the serum-free culture media used for the production of recombinant protein biopharmaceuticals. Low concentrations of methotrexate are used to exert a selective pressure favoring proliferation of the desired recombinant host cells engineered to over-express DHFR [2]. Methotrexate is pharmaceutically active and its removal from the purified drug product, confirmed by laboratory analysis, must be assured for safety reasons.

There are several high-performance liquid chromatography (HPLC) methods to determine methotrexate in blood serum and other physiological fluid matrices [3–6]. However, these methods generally require a solid-phase extraction or other sample clean-up and/or concentration step. For detecting residual methotrexate in protein pharmaceuticals with high sensitivity we sought, ideally, a direct single-column HPLC analysis without sample clean-up steps or other potentially problematic sample pretreatments.

Shielded-hydrophobic-phase HPLC (SHP-HPLC) was developed for this very purpose [7–9] and employs a restricted-access hydrophobic bonded-phase that is shielded by a hydrophilic outer layer. Small solutes, such as methotrexate, penetrate the hydrophilic outer network and are retained on the column. Large protein molecules are shielded (physically excluded) from contact with the hydrophobic surface and are not retained. Clogging of the column by protein is thus prevented and direct injection of

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biological samples greatly facilitated. In this paper, we describe the validation of a SHP-HPLC method for determining residual methotrexate in a recombinant IgG1 monoclonal antibody pharmaceutical [10]. The validation of the method included accuracy, precision, selectivity, limit of detection (LOD), limit of quantitation (LOQ), linearity range, ruggedness, robustness and stability.

2. Experimental

2.1. Materials

Ammonium acetate (analytical-reagent grade) and glacial acetic acid (USP grade) were obtained from Mallinckrodt. Acetonitrile (HPLC grade) was from Burdick and Jackson. Methotrexate was from Fluka. A Hisep SHP column was purchased from Supelco.

2.2. Procedure

2.2.1. Preparation of standard

Approximately 5 mg of methotrexate was weighed and transferred into a 100-ml volumetric flask. A 20-ml volume of acetonitrile and 20 ml of 20 m*M* ammonium acetate (pH 4.0) buffer was added and dissolved by sonication for approximately 3 min. The volume was raised to 100 ml with buffer to produce a stock solution containing 50 μg/ml of methotrexate. This solution was diluted 100-, 200-, 500-, 1000- and 2000-fold with buffer to produce 0.5 μg/ml, 0.25 μg/ml, 0.1 μg/ml, 0.05 μg/ml and 0.025 μg/ml solutions of methotrexate, respectively. The stock solutions were covered with aluminum foil and stored at 4°C in the dark for up to two weeks.

2.2.2. Preparation of sample and methotrexate spiked sample

The original protein sample was reconstituted or diluted to 20 to 25 mg/ml. A series of spiked samples was prepared by mixing 400 μl of the protein sample with 400 μl each of the 0.5, 0.05 and 0.025 μg/ml methotrexate standard solutions in separate tubes.

2.3. Chromatographic conditions

A Supelco Hisep SHP column (5-μm particles, 15 cm×4.6 mm I.D., part No. 5-8935) was employed and run under isocratic conditions. The mobile phase was 100 m*M* ammonium acetate, 10% acetonitrile adjusted with glacial acetic acid to pH 4.0. The flow-rate was 1.0 ml/min and UV detection was at 300 nm. Run time was 15 min and column temperature set at 45°C. The column was equilibrated at least 15 min before injecting sample. A blank was run prior to injecting standard, and additional blanks between the standards and sample. Volumes of 100 μl of the standards and the protein were injected in triplicate. Volumes of 200 μl of methotrexate spiked sample solutions were injected in triplicate. The void volume time, used to calculate k' values, was the time from injection to the first baseline deflection.

3. Results and discussion

Fig. 1 shows the SHP-HPLC of methotrexate spiked at 0.025 μg/ml into a 20 mg/ml protein sample (IgG1 antibody) on the Hisep column. The small peak for residual methotrexate is sufficiently resolved from the massive protein peak eluting with the solvent front.

3.1. Range and accuracy

The method calibration with aqueous methotrexate standards at 0.025, 0.05, 0.1, 0.2, 0.25 and 0.5 μg/ml gave typical values for slope and intercept of $1.0 \cdot 10^5$ μV s ml/μg and $-1.0 \cdot 10^3$ μV s, respective-

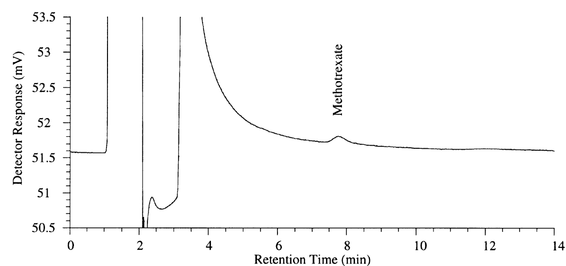

Fig. 1. SHP-HPLC (Hisep column) of a recombinant IgG1 antibody (20 mg/ml protein) spiked with 0.025 μg/ml methotrexate.

Table 1
Summary of accuracy study

Amount spiked (μg/ml)	Lyophilized protein recovery (%)	Lyophilized protein area response R.S.D. (%)	Bulk biological substance recovery (%)	Bulk biological substance R.S.D. (%)
0.025	111.7	3.51	110.9	3.26
0.05	107.5	3.2	94.5	2.97
0.1	103.2	3.22	98.9	4.15
0.25	92.3	0.97	100.9	4.74

ly, with a correlation coefficient of the linear regression curve (R^2) of 0.999. Using a standard curve with the linear range of 0.025 μg/ml to 0.5 μg/ml methotrexate, the recovery at 0.025 μg/ml was found to be within the range of 90–120% with relative standard deviation (R.S.D.) ≤10%. The higher concentrations were found to be in the range of 90–110% with R.S.D.≤5% (Table 1). The recoveries in Table 1 for the lyophilized protein test article (final drug substance) show an apparent trend versus concentration. This is only an apparent trend that occurred in this particular validation experiment as the recoveries across many dozens of analyses (data not shown) are essentially random.

3.2. Precision

Table 2 shows the intra-assay precision within one day. Table 3 shows inter-assay precision over multiple days. Table 4 shows inter-assay precision for two analysts using two different instruments. These data show that the R.S.D. at the LOQ (0.025 μg/ml) was less than 5% and the R.S.D. at higher concentrations was less than 3%. The data were compared statistically using the *F*- and *T*-tests and the results showed no difference between analysts. The precision of assay was found to be valid on both LC systems.

3.3. Detection and quantification limits

Table 5 shows determined noise is 227 μV s which comes from a ''blank'' baseline. The detection limit was estimated as three times the 227 μV s noise, which is 681 μV s. The LOD is 0.01 μg/ml after the conversion of μV s to concentration. The area of spiked protein sample (0.01 μg/ml) to detected noise ratio (S/N) is greater than 3 (Table 6). The LOQ was estimated as 10-times the determined noise (0.023 μg/ml). The LOQ was found to be 0.025 μg/ml by experiment. At 0.025 μg/ml, the analyte recovery for the biopharmaceutical sample was 107% of the theoretical with R.S.D.s≤10% in all instances.

Table 2
Repeatability of three methotrexate standards

Solution	Standard concentration (ng/ml)	Average area for 6 injections (μV s)	R.S.D. (%)
A	25.15	2788	4.43
A	50.30	5706	2.27
A	100.60	11 612	1.76
B	25.15	2823	3.3
B	50.30	5872	2.56
B	100.60	12 273	1.32
C	25.15	2721	2.32
C	50.30	5383	2.53
C	100.60	11 836	2.78

Three separate standard preparations (A, B, C) were used.

Table 3
Inter-assay precision of methotrexate standard over three days

Assay day	Methotrexate concentration	Average response for 6 injections (μV s)	R.S.D. (%)
22 May	0.025	2788	4.43
02 Jun	0.025	2407	4.68
17 Jun	0.025	2782	4.66
	Mean	2659	
	% R.S.D.:	8.20	
22 May	0.05	5706	2.27
02 Jun	0.05	4961	2.71
17 Jun	0.05	5438	2.96
	Mean:	5368	
	% R.S.D.:	7.00	
22 May	0.1	11 612	1.76
02 Jun	0.1	10 717	1.54
17 Jun	0.1	11 305	1.75
	Mean:	11 211	
	% R.S.D.:	4.00	

3.4. Ruggedness

The reproducibility of methotrexate assay is rugged with respect to vendor-to-vendor differences of methotrexate. The recovery of spiked sample at 0.025 μg/ml was found to be within 95–105% of theoretical with R.S.D.≤5%. The higher concentrations were recovered in the range of 98–100% with R.S.D.≤2%. Table 7 shows the reproducibility of methotrexate assay is also rugged with respect to and column-to-column differences for same column vendor or different column vendors.

3.5. Robustness

Table 8 shows that the assay is robust when

Table 4
Inter-assay precision of methotrexate standards by two analysts using two instruments

Analyst	Instrument	Methotrexate (μg/ml)	Average area for 6 injections (μV s)	R.S.D. area (%)
1	Beckman 126	0.025	2245	4.83
1	Beckman 126	0.05	5026	1.54
1	Beckman 126	0.1	10 527	2.60
2	Beckman 126	0.025	2550	4.68
2	Beckman 126	0.05	5534	1.76
2	Beckman 126	0.1	11 371	1.82
1	HP 1100	0.025	4437	1.03
1	HP 1100	0.05	10 081	1.67
1	HP 1100	0.1	22 583	2.17
2	HP 1100	0.025	5677	1.04
2	HP 1100	0.05	11 468	1.36
2	HP 1100	0.1	24 121	1.66

Table 5
Estimation of limit of detection from ''blank'' baseline

	Day	Injection No.	LC No. 1 6.5–8.1 min	LC No. 1 10.7–13.0 min	LC No. 2 6.5–8.1 min	LC No. 2 10.7–13.0 min
Daily average area	1	5	247	345	283	493
Daily S.D.	1	5	48	152	114	233
Daily average area	2	5	282	394	230	547
Daily S.D.	2	5	132	261	71	223
Daily average area	3	5	209	314	230	508
Daily S.D.	3	5	91	291	99	390
S.D. of LC Nos. 1 and 2 on 3 different days:						227
3S.D.:						681
10S.D.:						2270

An estimate of determined noise was made by injecting three replicates of protein sample formulation buffer on three different days using two different detectors with two different Beckman 126 pumps. The area values in the table are in μV s.

Table 6
Determination of limit of detection from ''blank'' baseline

Spiked (μg/ml)	Assay	Area of spiked peak response (μV s)	Determined noise	Signal-to-noise ratio
0.01	Day 1	1041	227	5
	Day 1	1345	227	6
	Day 1	1182	227	5
0.01	Day 2	1392	227	6
	Day 2	1150	227	5
	Day 2	1380	227	6
0.01	Day 3	1305	227	6
	Day 3	1705	227	8
	Day 3	1187	227	5

method parameters were varied within the specified ranges. No single parameter, extended to the specified limits, resulted in a dramatic adverse affect on the system suitability. The data also show that if the temperature of column is increased, the capacity factor (k') will decrease. If the flow-rate of mobile phase is increased, the retention time, number of theoretical plates, and area response will decrease.

Table 7
Multiple column qualification results (n=3)

Column					
Vendor	Lot No.	Tailing	R.S.D. (area of replicates) (%)	Column efficiency (plates)	R.S.D. (elution time of replicates) (%)
Supelco	207293AB	1.30	0.16	1544	0.12
Supelco	207289AB	1.35	0.93	1371	0.11
Regis	R9-156-1	0.97	0.37	4435	0.00
Regis	R9-283-1	0.89	0.01	3705	0.23

Table 8
Robustness experimental data

Run	Day	T (°C)	Flow-rate (ml/min)	Acetonitrile (%)	λ_{max} (nm)	Elution time (min)	R.S.D. of elution time (%)	Area (μV s)	R.S.D. of area (%)	Plates	Tailing	k'
11	2	50.0	0.90	12.0	295	7.98	0.00	63 487	0.84	1898	1.27	3.70
10	2	50.0	0.90	8.0	305	8.13	0.00	66 194	0.11	1984	1.25	3.78
2	1	40.0	1.10	8.0	305	7.50	0.00	53 232	0.80	1323	1.31	4.37
4	1	40.0	1.10	12.0	295	7.22	0.00	51 769	0.51	1257	1.27	4.23
5	1	45.0	1.00	10.0	300	7.42	0.00	60 688	0.37	1586	1.30	3.78
6	1	45.0	1.00	10.0	300	7.42	0.00	60 174	0.43	1596	1.27	3.78
7	2	45.0	1.00	10.0	300	7.38	0.00	59 369	0.36	1607	1.29	3.76
8	2	45.0	1.00	10.0	300	7.38	0.00	59 678	0.50	1610	1.29	3.76
1	1	40.0	0.90	8.0	295	9.29	0.10	61 802	0.23	1518	1.33	4.37
9	2	50.0	1.10	8.0	295	6.62	0.00	51 844	0.87	1788	1.22	3.79
3	1	40.0	0.90	12.0	305	8.86	0.11	63 941	0.52	1473	1.32	4.21
12	2	50.0	1.10	12.0	305	6.50	0.00	54 619	0.28	1694	1.25	3.64

The range of the method parameters varied were column temperature, 45±5°C; flow-rate, 1.0±0.1 ml/min; percent acetonitrile of mobile phase, 10±2% and the wavelength of detection, 300±5 nm. Values are averages for triplicate injections.

Variance in temperature and flow-rate should be closely monitored.

3.6. Stability

The stability of the standards, eluent, and samples were investigated by studying the chromatographic suitability and linearity of the methotrexate standards at time points up to 14 days. The stability of the standards and samples was investigated by studying the accuracy of the assay up to 72 h. It was found that the assay is stable using a 0.5 μg/ml methotrexate standard peak at room temperature in a clear autosampler vial for up to 72 h. It is also demonstrated that the assay is stable with HPLC eluent that is 14 days old. However, the assay may show some adversely biased accuracy results in protein samples after 48 h. Therefore, caution should be taken when preparation of spiked samples, their storage and actual analysis time is considered. Methotrexate decomposes in aqueous solution by both thermal and photolytic routes [11–13].

4. Conclusions

The assay to determine methotrexate by SHP-HPLC is a selective, sensitive, and reproducible method in which trace levels of methotrexate in recombinant protein biopharmaceutials can be separated from protein matrix and quantified. The LOD is as low as 0.01 μg/ml. The quantitation limit is 0.025 μg/ml. Methotrexate is fully resolved from biopharmaceutical sample matrices. The accuracy study shows the spike recoveries at LOQ are within the range of 90–120%. At higher concentrations, recoveries are within 90–110% with R.S.D.s≤5%. The precision study shows that the R.S.D. at LOQ is ≤5% and the R.S.D. at higher concentration is ≤3%. The assay to determine residual methotrexate is robust and rugged when method parameters and normal assay variables are within specified ranges, but variance in temperature and flow-rate should be monitored closely. Methotrexate standard can be kept in a clear autosampler vial up to 72 h without adverse effects on the assay. Eluent can be stored up to 14 days under ambient conditions.

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