Impurity Profiling with the Agilent 1200 Series LC System

Part 4: Method Validation of a Fast LC Method

Application Note

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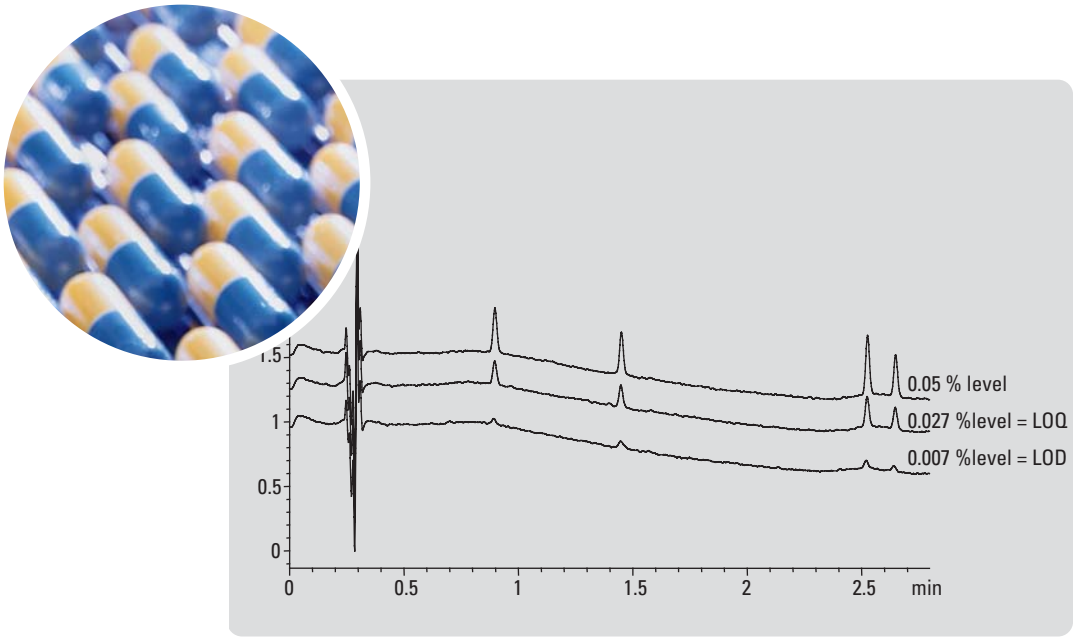

Abstract

Analytical laboratories working in a regulated environment have to validate their methods, to ensure that results fulfill all regulatory requirements. The validation procedure introduced in this Application Note was based on recommendations from the U.S. Pharmacopeia (USP) and the ICH guidelines Q2B respectively. A fast LC method for one main compound and its four impurities is successfully validated.

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Introduction

Analytical laboratories working in a regulated environment must validate their methods in order to ensure that the results fulfill all regulatory requirements. In addition, the results from different users in different laboratories are comparable, even though separate equipment was used. Consequently, the method is required to be as robust as possible to compensate for variations, which might occur if different users perform the same analysis on the same or different equipment. The validation procedure introduced in this Application Note was based on recommendations from the U.S. Pharmacopeia (USP), ICH guidelines Q2B[1,2,] as well as FDA guidelines[4,5], which are recognized worldwide and employed by analytical laboratories in the pharma, food, environmental and chemical industry. In the following experiment, a fast LC method for one main compound and its impurities is validated, (see figure 1 for a sample chromatogram). Method parameters were obtained from the method development group (reference 3).

Experimental

An Agilent 1200 Series Rapid Resolution LC system was used with the following modules:

- Agilent 1200 Series binary pump SL and vacuum degasser
- Agilent 1200 Series high-performance autosampler SL
- Agilent 1200 Series thermostatted column compartment SL
- Agilent 1200 Series diode-array detector SL
- Data acquisition and evaluation software: Agilent ChemStation B.02.01.SR1
- ZORBAX SB C-18 RRHT columns with internal diameters of 4.6 mm and lengths of 50 mm, packed with 1.8-µm particles
- Main compound and pure impurities were obtained using the purification procedure decribed in reference 6.

Validation procedure

Having done some pre-validation experiments, the following validation protocol for the above-described method[3] was set up:

Validation protocol :

1. Precision of areas and RT of the main compound	6 concentrations, 6 runs each
2. Accuracy of main compound	6 concentrations, 6 runs each
3. Linearity of main compound	6 concentrations, 6 runs each
4. Carry over for main compound	3 injections of stock solution
5. Range of main compound	
6. Precision of areas and RT of impurities	7 concentrations, 6 runs each
7. Accuracy of impurities	7 concentrations, 6 runs each
8. Linearity of impurities	7 concentrations, 6 runs each
9. Range of impurities	
10. Limit of Detection and LOQ	
11. Robustness of main compound and impurities	Different column temperatures, flow rates, injection volumes, TFA concentrations, gradient steepness, wavelength, users and instruments, no ruggedness tests

Specificity was tested and is given, see reference 3. No further sample preparation steps were taken. The sample compounds were weighed and dissolved in water.

Results and discussion

Validation of main compound
Sample preparation:
The stock solution of the main compound contained 2.3 mg/mL. This solution was diluted to give the following desired concentrations:

Main compound:

- Stock solution: 2.3 mg/mL (used for carry over and linearity tests)

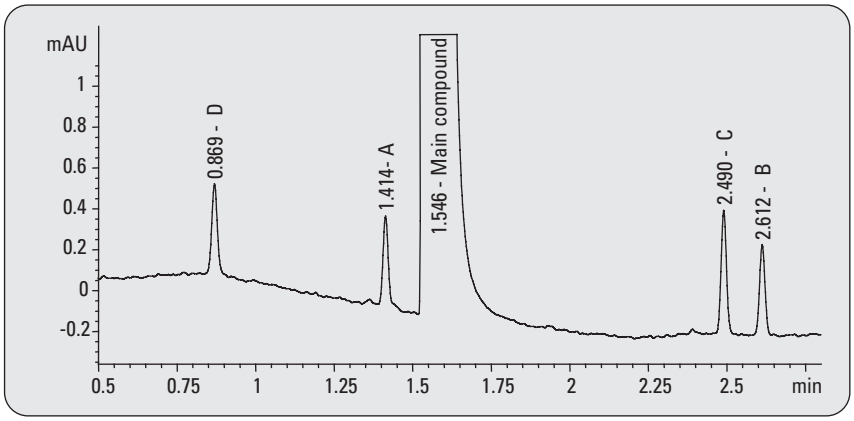

Figure 1
Analysis of the main compound and its 4 impurities. Main compound 1.15 mg/mL, impurities 0.072 % of the main compound.

- Dilution 1: 1.15 mg/mL (used for precision, accuracy, linearity and robustness tests)
- Dilution 2: 0.575 mg/mL (used for precision, accuracy, linearity test)
- Dilution 3: 0.288 mg/mL (used for precision, accuracy, linearity test)
- Dilution 4: 0.144 mg/mL (used for precision, accuracy, linearity test)
- Dilution 5: 0.072 mg/mL (used for precision, accuracy, linearity test)

1. Precision of retention times and areas

The results for retention time and area precision for all different concentration levels are summarized in figure 2. The precision limit for retention times is 0.1 % rsd. The precision limit for areas is 2 % rsd. For all concentrations the limits for retention time and area precision are fulfilled. To achieve sufficient resolution from the impurities, the concentration of the main compound should be <1.2 mg/mL.

2. Accuracy of main compound

The accuracy was tested using the above-mentioned concentrations. A maximum deviation of 2 % was set as the limit. All concentrations passed the requirement, (figure 3).

3. Linearity for main compound

The linearity was tested using all 6 concentrations. A correlation coefficient of > 0.99990 was set as the limit for this concentration range. The determined correlation coefficient was 0.99999. The response factors are within the 5 % limit from 2.3 down to 0.073 mg/mL (figure 4).

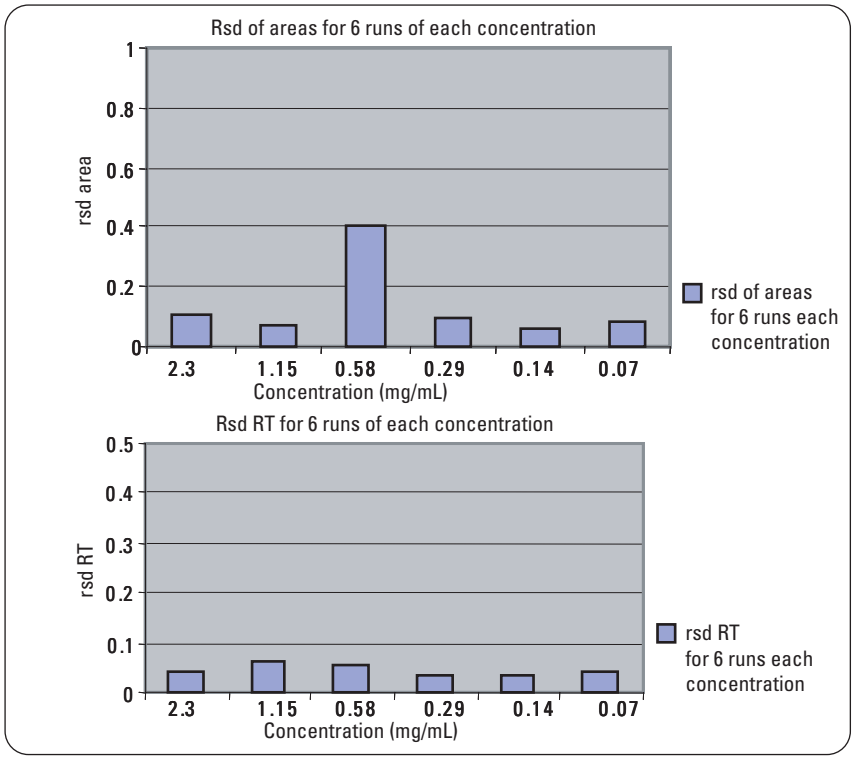

Figure 2
Precision of retention times and areas of different concentrations of the main compound; 6 runs for each concentration.

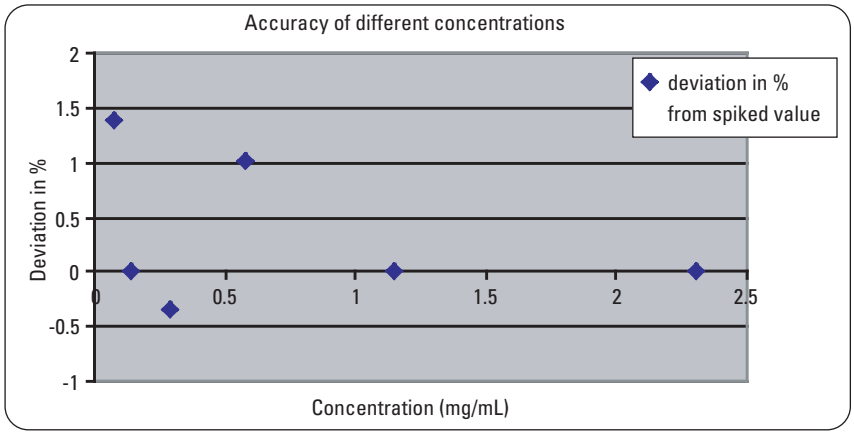

Figure 3
Accuracy for different concentrations of the main compound, 6 runs for each concentration.

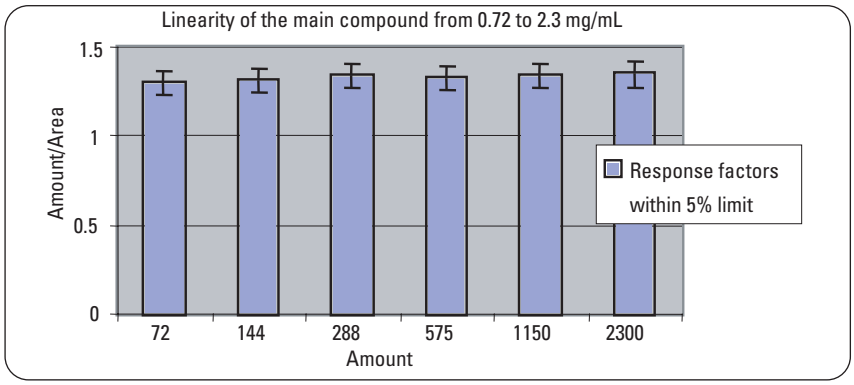

Figure 4
Linearity of the main compound.

4. Carry-over of main compound

The carry-over was evaluated by injecting the stock solution 6 times followed by the injection of 5 µL pure water. The carry-over was found to be ~ 0.01 %.

5. Range of main compound

The range for the main compound with good precision, accuracy and linearity lies between 2.3 down to 0.073 mg/mL.

Validation of impurities A, B, C, and D

Sample preparation

The impurities were analyzed by preparing different concentration levels using the pure impurity compounds. The stock solution contained 4.9 mg/mL for impurity A, C, and D and 4.6 mg/mL of compound B. This solution was diluted by a factor of 1:1000 to obtain a concentration in the µg/mL range. The performance was evaluated for 7 impurity concentrations based on the diluted mixtures, see table 1.

6. Precision of retention times and areas of impurities

Precision of areas was evaluated for 6 concentrations from 0.153 up to 4.9 µg/mL for compounds A, C and D, and from 0.144 to 4.6 µg/mL for compound B. Precision of retention times was evaluated for all 7 concentrations. A summary of all results is shown in figure 5. The 0.05 % level fulfilled the acceptable limit of < 5 % rsd for areas. The 0.027 % level showed an area precision < 6 % rsd, which is within the 10 % limit.The 0.013 % level showed an area precision of < 14 %, which is acceptable for this low concentration. Retention time precision is below 0.5 % rsd for all

Impurity	Dilution 6 µg/mL (% level)*	Dilution 5 µg/mL (% level)*	Dilution 4 µg/mL (% level)*	Dilution 3 µg/mL (% level)*	Dilution 2 µg/mL (% level)*	Dilution 1 µg/mL (% level)*	Stock solution µg/mL (% level)*
A	0.077 (0.007%	0.153 (0.013%)	0.306 (0.027%)	0.613 (0.05%)	1.225 (0.107%)	2.45 (0.213%)	4.9 (0.426%)
B	0.072 (0.006%)	0.144 (0.013%)	0.287 (0.025%)	0.575 (0.05%)	1.150 (0.1%)	2.30 (0.2%)	4.6 (0.4%)
C	0.077 (0.007%	0.153 (0.013%)	0.306 (0.027%)	0.613 (0.05%)	1.225 (0.107%)	2.45 (0.213%)	4.9 (0.426%)
D	0.077 (0.007%	0.153 (0.013%)	0.306 (0.027%)	0.613 (0.05%)	1.225 (0.107%)	2.45 (0.213%)	4.9 (0.426%)

* Percentage is based on a main compound concentration of 1.15 mg/mL

Table 1
Dilution series for impurities.

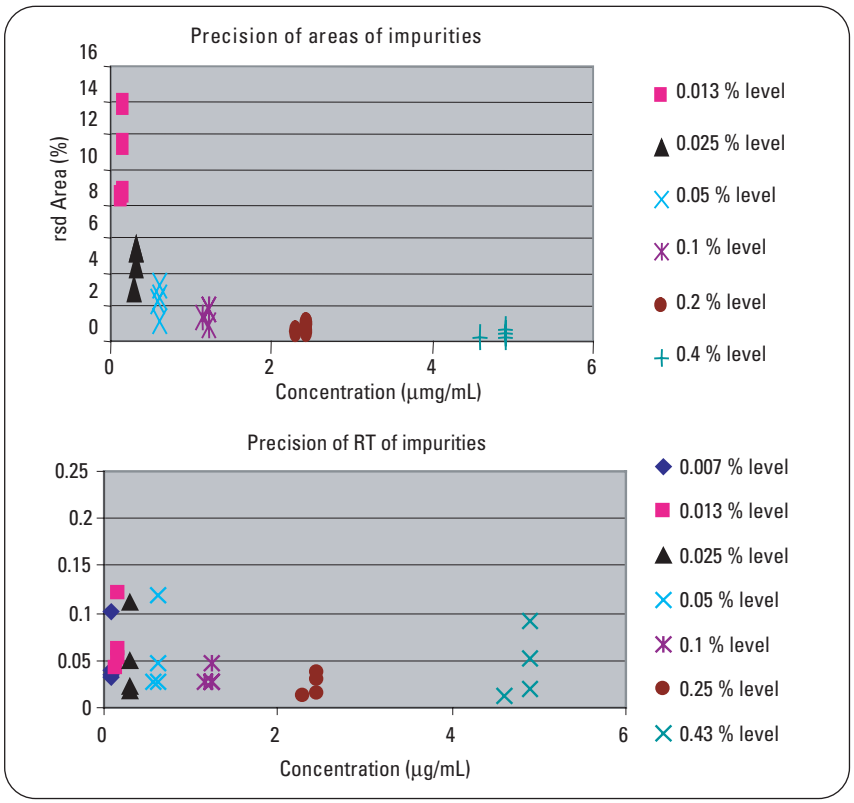

Figure 5
RSD of retention times and areas for impurities; 6 runs for each concentration .

concentrations and passed the set limit of rsd < 0.5 %. An example of the precision of retention times and areas is shown in figure 6. Ten chromatograms from 10 consecutive runs were superimposed and the precision of retention times is for all compounds < 0.16 %.

7. Accuracy of impurities

Accuracy of impurities was evaluated for the 0.027 %, 0.05 %, 0.107 % and 0.213 % level of impurities A, C and D. The 0.025 %, 0.05 %, 0.1 % and 0.2 % level of impurity B was evaluated. Spiked samples with known concentrations were analyzed and data were evaluated using auto-integration and the calibration parameters used for precision measurements. The deviation from the spiked value should not be more than ± 5 %. The maximum deviation is ± 4.3 % for the determined concentration ranges (figure 7). Each concentration was injected 6 times and the average value was used as the calculated amount.

8. Linearity of impurities

The linearity of all impurities was tested using all 7 concentration levels. A correlation coefficient of > 0.9990 was set as the limit for this concentration range. The established correlation was 0.9998. Linearity based on the response factors is calculated between 4.9 µg/mL (impurities A, C, and D), 4.6 µg/mL (impurity B) down to 0.306 µg/mL (impurities A, C, and D), and 0.287 µg/mL (impurity B); an example is given in figure 8. The response is within the 5 % limit for these concentration ranges.

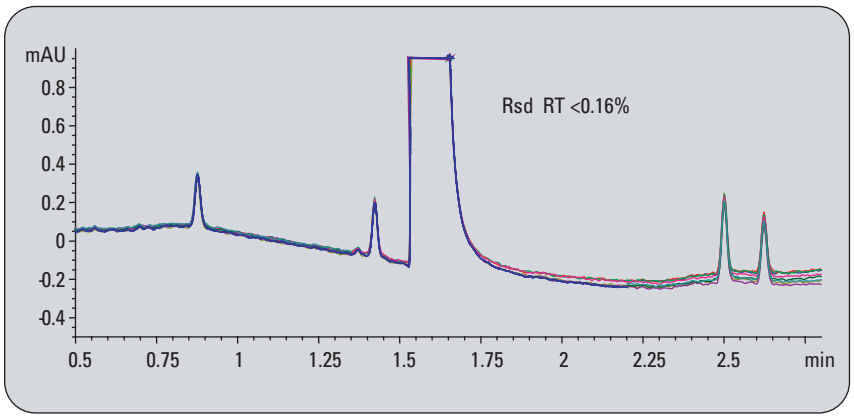

Figure 6
Overlay of 10 chromatograms within 1 sequence, RSD RT for all peaks < 0.16 %.

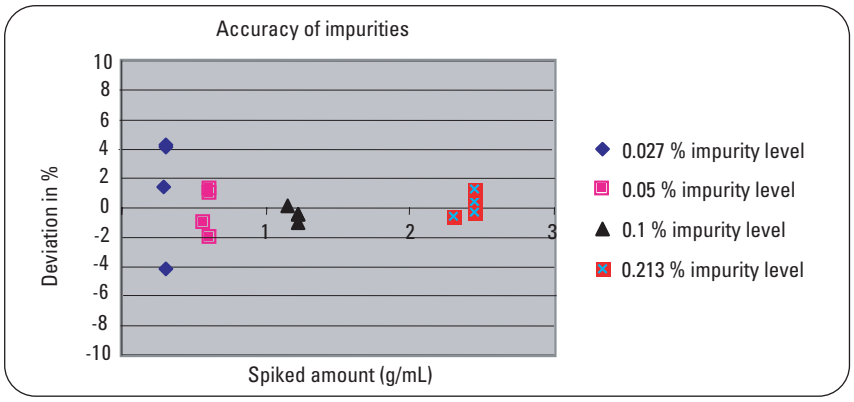

Figure 7
Determination of accuracy of impurities A, B, C, and D. 6 runs for each concentration with maximum deviation ± 4.5 % for the 0.027 % level.

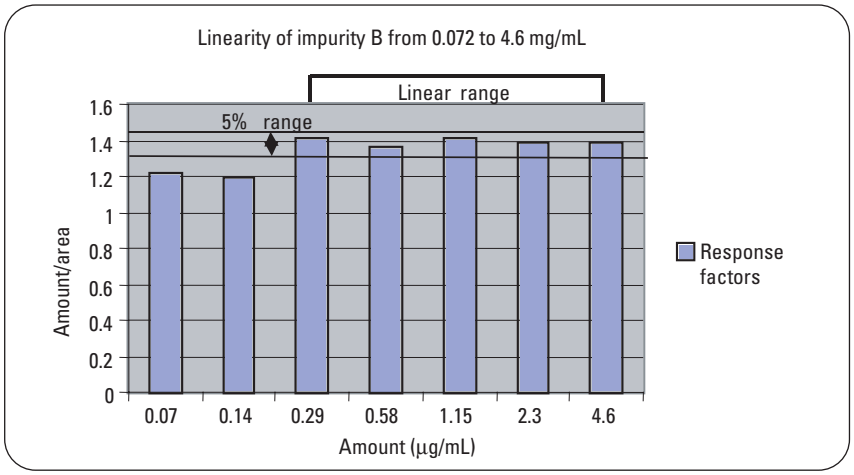

Figure 8
Linearity of impurity B across a concentration range between 0.077 to 4.9 µg/mL.

9. Range of impurities

The range of impurities with an acceptable precision, accuracy and linearity is between 0.306 µg/mL (0.287 µg/mL impurity B) and 4.9 µg/mL (4.6 µg/mL impurity B).

10. Limit of detection (LOD) and limit of quantitation (LOQ) for impurities

The limit of detection was determined using the 0.007 % level of compounds A, C and D. For compound B the 0.006% level was used. In figure 9 the resulting chromatogram, lower trace, is shown. All impurities at that concentration level have a signal to noise ratio S/N > 2 (table 2).
In figure 9 chromatograms of 2 further concentration levels for the impurities related to the main compound concentration of 1.15 mg/mL are shown. The trace in the middle shows the 0.027 % level (impurities A, C andD) and the 0.025 % level (impurity B), which is the limit of quantitation, and the upper trace shows the 0.05 % level. In table 2 the results for LOD and LOQ are summarized. The limit of quantitation was evaluated for the 0.027 and the 0.025 % concentration levels respectively. The signal to noise ratio limit for the LOQ is 10 and the values shown for the 0.027 and 0.025 % levels are proximate. The 0.05 % is clearly above the set limit. The area precision for the 0.05 % level is < 2.6 %, and for the 0.027 and 0.025 %levels the area precision is < 5.4 %.

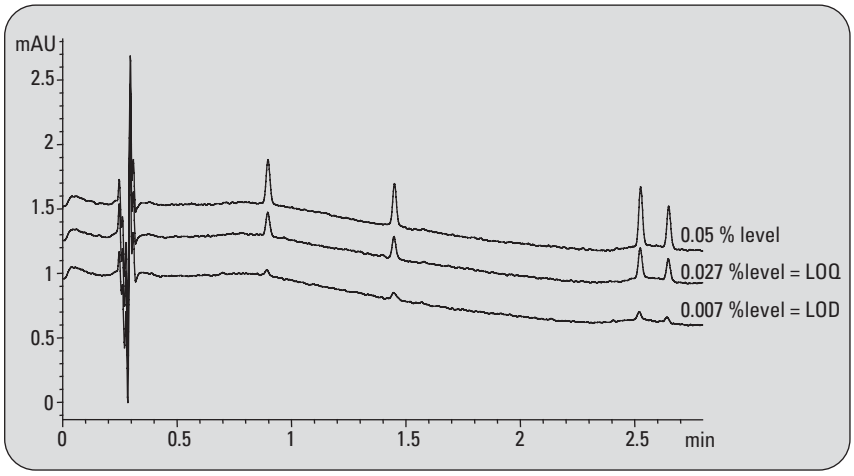

Figure 9
Chromatogram of impurities close to the limit of detection at the 0.00 7 % level (lower trace). The trace in the middle shows the chromatogram at the 0.027 % level, close to the limit of quantitation. The upper trace shows a chromatogram of the 0.05 % level.

Impurity concentration	LOD	S/N	LOQ	S/N
A	0.007 % level	2.8	0.027 %level	9.5
B	0.006 % level	2.1	0.025 %level	9.1
C	0.007 % level	3.8	0.027 % level	13.1
D	0.007 % level	3.4	0.027 %level	10.1

Table 2
LOD and LOQ for impurities.

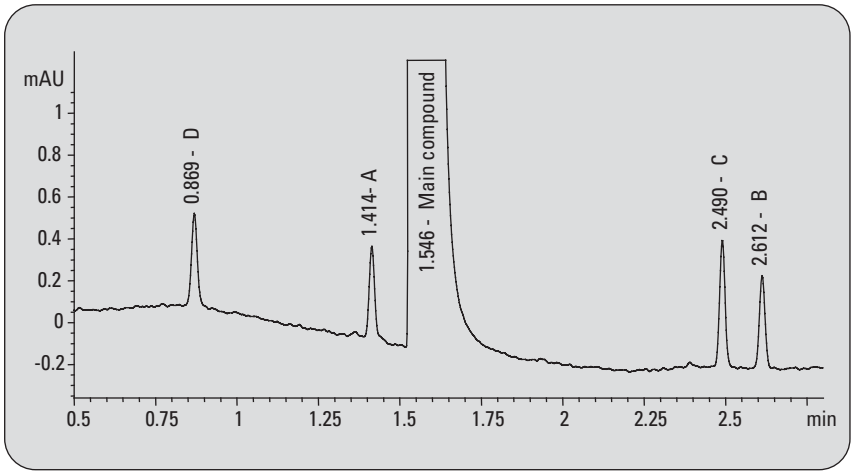

Figure 10
Chromatogram of sample used for robustness tests.

11. Robustness of method for main compound and its impurities

To test the robustness of the method, the main compound was dissolved in water with a concentration of 1.15 mg/mL. This solution was spiked with impurities to achieve an impurity concentration level of approximately 0.07 % (figure 10). In table 3 the results for the main compound and impurities are summarized. The only critical parameter is the

wavelength. The wavelength should not vary more than 1 nm. All other parameter changes cause deviations for the areas of less than 2 %, which is acceptable for a main compound. The results for the impurities are also shown in table 3. All results except the wavelength change are within the 10 % limit for the areas. The wavelength should not vary more than 1 nm.

In figure 11 an example is shown for the day-to-day repeatability for retention times and areas. The results of 3 sequences are overlaid. Each sequence contained 10 runs and were analyzed on 3 consecutive days. The instrument was turned off overnight.

Parameter changed	Deviation of amounts for the main compound 9%)	Resolution between main compound and impurity A	Deviation of amounts (%) for impurities (spiked amounts fig. 10)
Flow ± 2 %	1.96 % rsd	2.56 for + 2 % change 2.55 for - 2 % change	< 9.8 % rsd for amounts
Column temperature ± 5 %	0.17 % rsd	2.61 for 5 % change 2.51 for + 5 % change	< 5 % rsd for amounts
Gradient slope ±10 %	0.07 % rsd	2.59 for + 10 %change 2.51 for - 10 % change	< 3 % rsd for amounts
Injection volume ± 5 %	0.001 % from expected amount	2.45 for + 5 % change 2.62 for - 5 % change	6.5 % from expected amount
TFA concentration ± 10 %	0.07 % rsd	2.49 for -10 % change 2.61 for +.10 %change	< 4 % rsd for amounts
Wavelength ± 3 nm	± 10 % for area counts	2.61	± 18 % for area counts
Day-to-day repeatability	0.34 % rsd for retention times	2.60-2.63	< 0.6 % for retention time
Day-to-day repeatability	0.35 % rsd for amounts and 1.15 mg/mL weighted sample	2.60 – 2-63	< 4.5 % for amounts
3 different instruments, different columns, intermediate precision	± 0.47 % deviation for 1.15 mg/mL	2.6 -3.1	± 10 % for 0.025 % level for amounts ± 4.5 % for 0.05 % level for amounts

Table 3
Robustness test results for main compound and impurities.

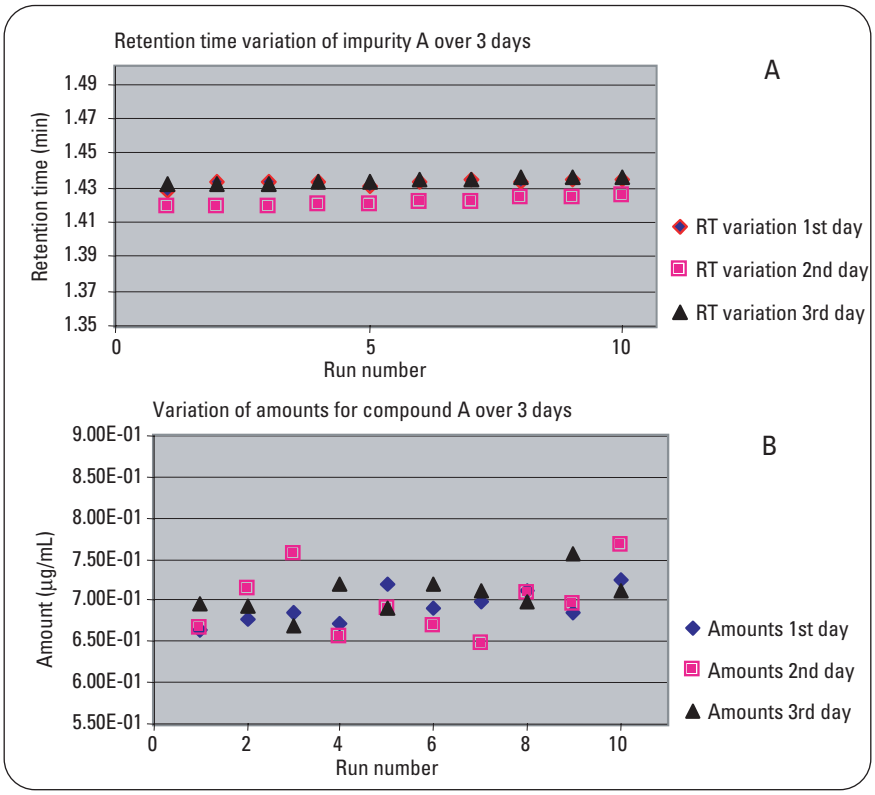

Figure 11
Day-to-day precision of retention times and areas, overlay of 3 sequences with 10 runs each. Repeatability of retention time day-to-day (figure 11a) < 0.6 %, repeatability for areas < 4.5 %, difference between highest and lowest amount < 16 % for the amounts (figure 11b).

Results of method validation

In table 4 the results of the method validation are summarized. The set limits are fulfilled. Special attention is required for the wavelength. The wavelength variation should not be more than ± 1 nm. Typically, a wavelength variation of ± 3 nm is considered acceptable. In this experiment, the limits for wavelength variations are more restrictive, based on the results.

Parameters	
Precision for areas	
• Main compound < 2 % for all experiments	passed
• Impurities < 10 % at the limit of quantitation	passed
Accuracy for main compound < ± 2 %	passed
Accuracy for impurities at LOQ ± 5 %	passed
Precision of Retention times < 0.5 % within 1 series	passed
Precision of retention time < 2 % day-to-day	passed
Linearity > 0.999	passed
Resolution > 2 for all peaks	passed
LOD S/N > 2 at the ~ 0.007 % level for impurities	passed
LOQ S/N > 10 at the ~ 0.027 % level for impurities	passed
Range of the main compound: 2.3 to 0.073 mg/mL	passed
Range of the impurities: 4.9 (0.426 % level) to 0.287 µg/mL (0.025 % level)	passed
Robustness tests for area deviation: < 2 % for main compound	passed*
Robustness tests for area deviation: < ± 5 % for impurities at the 0.05 %level	Passed*

*wavelength variations of ± 1 nm are acceptable and should be carefully controlled

Table 4
Results of method validation.

Conclusion

A fast LC method was developed for the analysis of a main compound and four impurities. The validation of this method was successful. All requirements regarding precision, linearity, accuracy and robustness were fulfilled. This signifies that the fast LC method can be used in QA/QC labs and is compliant with USP/ICH recommendations. Faster LC methods provide the same data quality and, as an additional benefit, higher sample throughput.

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