Application Note

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Simultaneous Impurity Analysis and Enantioseparation of Atenolol Using Achiral-Chiral 2D-LC

Author

Lucas Willmann
Agilent Technologies, Inc.

Abstract

To ensure the quality of drug products, chromatographic separation of synthetic byproducts from active pharmaceutical ingredients (APIs), as well as separation of enantiomeric variants of APIs are important tasks in the pharmaceutical industry. To enable impurity analysis by reversed-phase chromatography and subsequent enantioseparation of the chiral API in a single sample run, two-dimensional liquid chromatography (2D-LC) has been used for achiral-chiral separation of the beta-blocker (R/S)-atenolol. In the first chromatographic dimension, an Agilent InfinityLab Poroshell 120 EC-C18 column was used to separate (R/S)-atenolol from known impurities. In the second chromatographic dimension, an Agilent InfinityLab Poroshell 120 Chiral-T column was used for separation of R- and S-atenolol. The Agilent 1290 Infinity II 2D-LC System was used in high-resolution sampling mode, resulting in a highly reproducible separation in both chromatographic dimensions. The Agilent OpenLab CDS 2 Acquisition and Data Analysis Software with the Agilent 2D-LC Software Add-On enabled easy and fast method setup, as well as convenient analysis of the complex high-resolution sampling 2D-LC data.

Introduction

Analysis of pharmaceuticals with respect to potentially harmful impurities is an essential part of the drug development process, prior to market release. Impurities might not only be synthetic byproducts, but also enantiomeric variants of the API. Today, many chiral APIs are still administered as racemic mixtures, although there may be significant differences in the pharmacokinetic properties, and pharmacodynamic activity between enantiomers. Determination of enantiomeric impurities is particularly important with respect to the efficacy of a drug, because only one enantiomer might show a therapeutic effect, while the other enantiomer shows no effect or even toxic effects. Enantiopure drugs can potentially have benefits such as an improved therapeutic index, a faster onset of action, or a reduced propensity for drug-drug interactions.[1]

Chromatographic separation of chiral APIs, as well as their synthetic byproducts, can be a complex task and consequently requires sophisticated analytical techniques, such as two-dimensional liquid chromatography (2D-LC). The 1290 Infinity II 2D-LC System is an ideal tool to address this analytical challenge, as it enables application of conventional reversed phase column chemistries for achiral separation of byproducts in one chromatographic dimension and application of column chemistries for chiral separations in the other chromatographic dimension. To achieve this complex chromatographic separation, Agilent also offers a variety of superficially porous particle columns not only for conventional reversed-phase chromatography, but also for fast and high-efficiency chiral separations.[2] The Agilent 2D-LC Software for OpenLab CDS enables fast and easy method setup, as well as simple analysis of complex data even for nonexpert 2D-LC users.

Atenolol is a selective beta-adrenoreceptor agonist that is effective in patients with hypertension aiming towards prevention of heart attacks and strokes. It has a chiral center that is responsible for the existence of two different stereoisomers, (R)- and (S)-atenolol. It has been reported that the central hypotensive action of atenolol is selective for the (S)-enantiomer.[3,4] Nevertheless, atenolol is commercially available as racemic mixture.

This application note describes a 2D-LC workflow for simultaneous determination of atenolol impurities by reversed-phase chromatography as well as enantioseparation of (R/S)-atenolol using the 1290 Infinity II 2D-LC System. For this purpose, several software features of the Agilent 2D-LC Software for OpenLab CDS are highlighted.

Experimental

Instrument

- 2x Agilent 1290 Infinity II High-Speed Pump (G7120A) with Jet Weaver V35
- Agilent 1290 Infinity II Multisampler (G7167B) with Sample Thermostat (option #101)
- 2x Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- 2x Agilent 1290 Infinity II Diode Array Detector (G7117B) with 10 mm Max-Light Cartridge Cell (G4212-60008)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2D-LC Valve, Active Solvent Modulation (G4243A)
- 2x Agilent 1290 Infinity Valve Drive (G1170A) with Multiple heart-cutting (MHC) Valves equipped with 40 µL loops
- Agilent Single Quadrupole Mass Spectrometer (G6135C) with Agilent Jet Stream Source

Software

- Agilent OpenLab CDS Acquisition Software, revision 2.6 Update 4 or later versions
- Agilent 2D-LC Software for OpenLab CDS

Samples

Atenolol Impurity Standard – British Pharmacopeia Reference Standard (Sigma, Germany)

Columns

- **First dimension (^{1}D):** Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 150 mm, 2.7 µm (part number 693775-902)
- **Second dimension (^{2}D):** Agilent InfinityLab Poroshell 120 Chiral-T, 4.6 × 100 mm, 2.7 µm (part number 685975-603)

Chemicals

- **^{1}D Mobile phase A:** 20 mM ammonium formate in water (pH 4)
- **^{1}D Mobile phase B:** Methanol
- **^{2}D Mobile phase A:** Acetonitrile/methanol (6/4), 0.2% triethylamine, 0.3% acetic acid
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point of use cartridge (Millipak)
- Other mobile phase ingredients were obtained from Merck, Germany
- **Sample solvent:** Dimethylsulfoxide (DMSO) was purchased from Sigma, Germany

Enantiomeric excess (ee) was calculated in percent using the following formula, where A(R) is the peak area of the R-enantiomer and A(S) is the peak area of the S-enantiomer:

$$\% \text{ ee} = \frac{|A(R) - A(S)|}{A(R) + A(S)} \times 100$$

Method parameters

Parameter	Value
^{1}D Pump	
Flow	0.35 mL/min
Timetable	0 min: 2 %B 10 min: 30 %B 15 min: 60 %B 17 min: 90 %B 17.1 min: 2 %B
Pressure Limit	600 bar
2D-LC Settings	
Flow	1 mL/min
2D-LC Operation Mode	Heart-cutting (Time-based heart cut at 5.42 min, HiRes, Cut size: 5.5 s, Cuts: 5, Loop filling: 80%)
Gradient Phases	Analysis: 10 min, Equilibration: 0.1 min
Analytical Gradient	100 %A (isocratic)
Stop Time	66.52 min (For ^{1}D only runs the stop time was set to 20 min)
Flush Gradient	Use analytical gradient as flush gradient
Pressure Limit	400 bar
Multisampler	
Injection Volume	1 µL
Needle Wash	6 s in flush port, wash solvent: water:methanol (1:1)
Thermostat	Off
Column Compartments	
^{1}D Temperature	40 °C
^{2}D Temperature	20 °C
Diode Array Detectors (DAD)	
Wavelength	225 nm
Bandwidth	4 nm
Reference Wavelength	360 nm
Reference Bandwidth	100 nm
Peak Width	>0.013 min (0.25 s response time) (20 Hz)
Single Quadrupole (Used for ^{1}D Only Runs)	
Mass range	50 to 500 *m/z*
Polarity	Positive
Scan/Dwell time	500 ms
Fragmentor	135 V
Capillary Voltage	3,500 V
Nozzle Voltage	2,000 V
Gas Temperature	300 °C
Gas Flow	7 L/min
Nebulizer	45 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	11 L/min

Results and discussion

To achieve chromatographic separation of atenolol impurities and separation of the R- and S-enantiomers of atenolol in a single sample run, the 1290 Infinity II 2D-LC System was equipped with two superficially porous particle columns showing very different column chemistry: a C18, and a Teicoplanin bonded phase. Reversed-phase chromatography was performed in the first chromatographic dimension to separate known impurities from the atenolol peak. Application of the Agilent InfinityLab Poroshell 120 EC-C18 column resulted in excellent separation of compounds contained in the Atenolol Impurity Standard from British Pharmacopeia, while a moderate backpressure of up to 520 bar could be maintained throughout the total 1D-LC run time of 20 minutes (Figure 1).

The Agilent G6135C Single Quadrupole MS was used for one-dimensional LC/MS runs to identify the compounds of the impurity mixture. For 2D-LC runs, the MS was not used, in order to avoid unnecessary contamination with the mobile phase additive triethylamine used in the second chromatographic dimension. Atenolol impurities contained in the reference standard are depicted in Figure 2, showing their structure, molecular weight, and measured *m/z* value in positive ionization mode.

For enantioseparation of atenolol in the second chromatographic dimension, five consecutive cuts of 5.5-second duration were used to sample the entire (R/S)-atenolol peak, eluting in the first chromatographic dimension. OpenLab CDS Data Acquisition Software enabled fast and easy method setup for time-based high-resolution sampling using the 2D-LC preview panel, which is synchronized with the 2D-LC method parameters, and allows interactive cut editing based on imported chromatograms from the first chromatographic dimension.

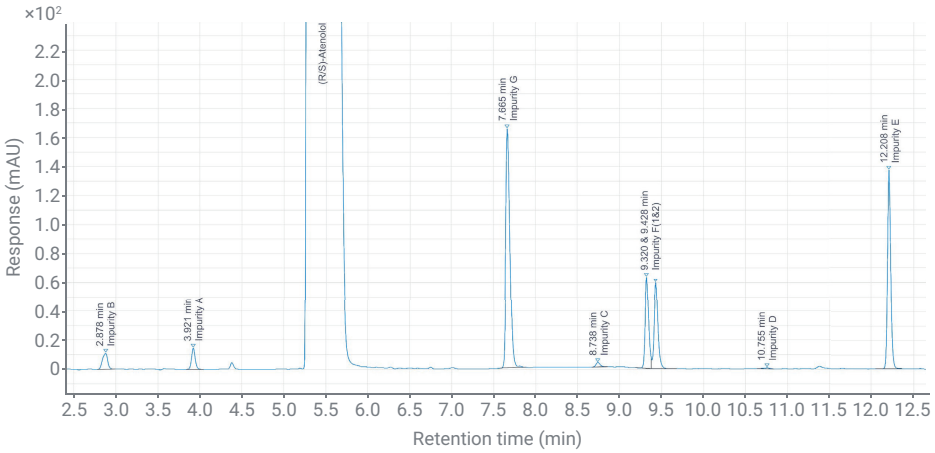

Figure 1. One-dimensional analysis of Atenolol Impurity Standard from British Pharmacopeia (10 mg/mL) showing a DAD signal at 225 nm.

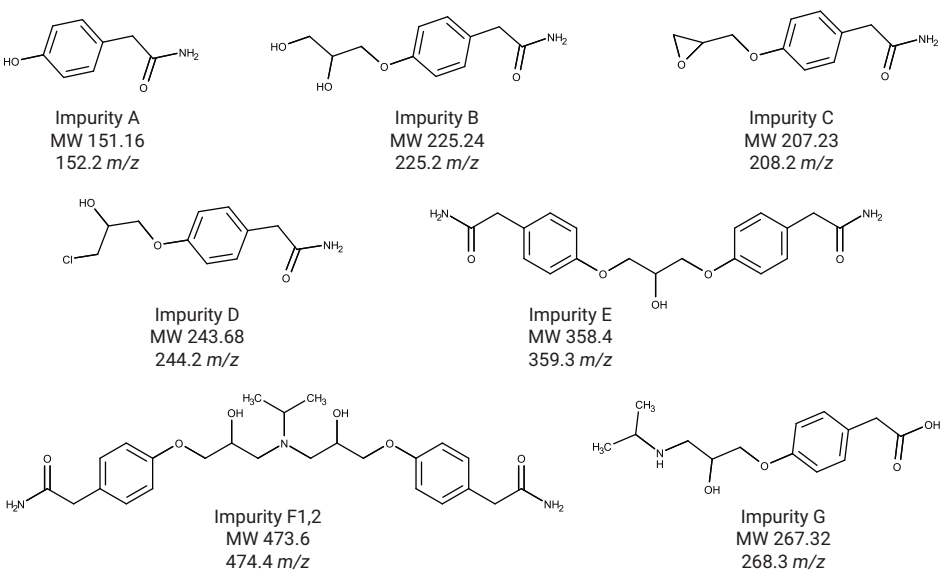

Figure 2. Atenolol impurities showing molecular structure, weight, and measured *m/z* in positive ionization mode.

Additionally, the stop time for 2D-LC runs is automatically calculated, taking the runtime of the first chromatographic dimension, the cut times, and the runtimes of all cut analyses into consideration. In OpenLab CDS 2D-LC Software, ^{2}D chromatograms can be easily displayed for data review by selecting the desired cuts in the ^{1}D chromatogram, the ^{2}D contour plot, or the sampling table. The sampling table is also an important tool to retrace the 2D-LC valve operation, showing several parameters, e.g. ^{1}D cut start times, ^{2}D run start times, and the parking location of cuts (Figure 3).

As shown in Figure 3, the cuts were analyzed in reverse order, starting with the fifth cut parked in the sixth loop, and ending with the first cut parked in the second loop. To eliminate run-to-run carryover resulting from remaining ^{1}D effluent, an automatic flush gradient was performed in the end of the run, using the first loop of MHC Deck A as starting position. As all cuts were parked in MHC Deck A, a system setup with only one MHC valve would also be a possible solution for the present workflow. To enable rapid data analysis, defined ^{2}D compounds can be selected in the injection results or ^{2}D contour plot, to show all relevant ^{2}D chromatograms simultaneously. The contour plots of a high-resolution sampling 2D-LC run are depicted in Figure 4. The Agilent 2D-LC Software for OpenLab CDS offers several visualization features for data analysis using the contour plot (e.g. highlighting of ^{2}D peak regions, modification of color scaling, and interpolation). The noninterpolated mode gives a better representation of the raw data and is beneficial for review of peak integration, boundaries, and assignment, while the interpolated mode is suitable for a comprehensive visualization of data and compounds.

Sampling Table

Cut selection | Signal selection

Cut #	^{1}D Cut start [min]	Cut size [s]	Trigger	^{2}D Run start [min]	Deck	Loop
1	5.42	5.50	Time	46.31	A	2
2	5.51	5.50	Time	36.21	A	3
3	5.60	5.50	Time	26.11	A	4
4	5.70	5.50	Time	16.01	A	5
5	5.79	5.50	Time	5.91	A	6

Figure 3. Sampling table resulting from 2D-LC analysis of atenolol.

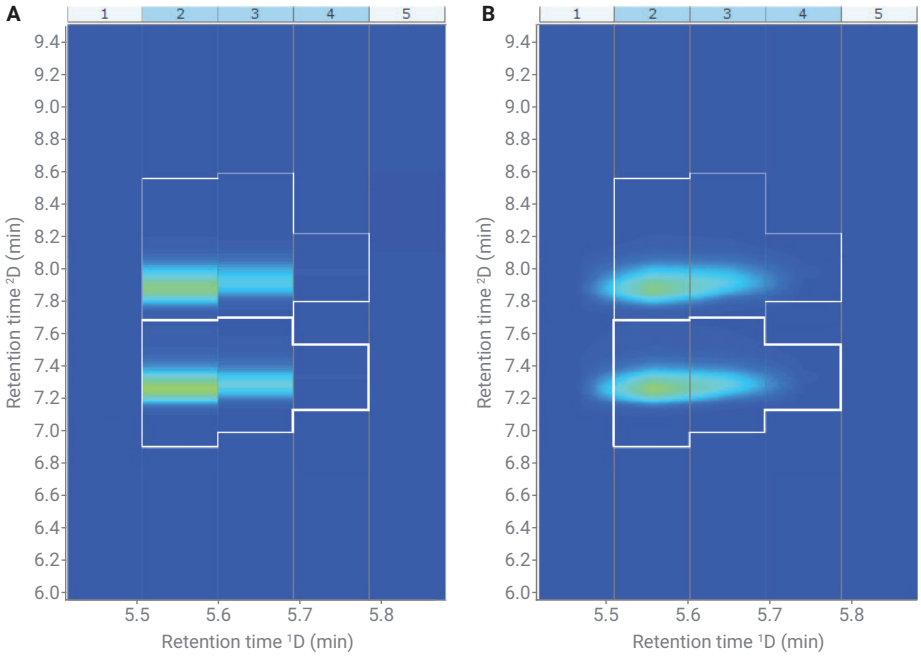

Figure 4. 2D-LC analysis of atenolol by high-resolution sampling showing ^{2}D contour plots of enantioseparated (S)- and (R)-atenolol in noninterpolated (A) and interpolated mode (B).

For enantioseparation in the ^{2}D, a teicoplanin bonded phase was used in polar organic mode. Teicoplanin columns are glycopeptide-based chiral columns, and exhibit several functional groups that can be neutral, positively, or negatively charged depending on the pH value of the mobile phase. Triethylamine was added to the mobile phase as a competitive organic amine to improve the peak shape, and acetic acid was added to ionize analytes for better chiral recognition and interaction with the chiral selector. Application of the Agilent InfinityLab Poroshell 120 Chiral-T column resulted in sufficient separation of (S)- and (R)-atenolol at a moderate backpressure of approximately 80 bar. Figure 5 shows cuts 2 to 4 of the second chromatographic dimension as conventional ^{2}D chromatograms in overlaid display mode. The (S)- and (R)-atenolol peaks were only found in cuts 2 to 4, indicating a sufficiently wide cut window for high-resolution sampling. A minimum resolution of 2.1 could be achieved for the separation of (S)- and (R)-atenolol, indicating baseline separation between the two neighboring peaks throughout all cuts.

As system suitability tests (SST) are generally performed during routine drug analysis to evaluate suitability and effectiveness of the complete chromatographic system, OpenLab CDS 2D-LC Software offers easy access to several parameters relevant for SST. SST parameters such as resolution, selectivity, number of theoretical plates, peak symmetry, or tailing can be displayed for 2D-LC runs using the peak table 2D (Figure 6). The ^{2}D retention time of a ^{2}D compound is calculated as the weighted average by area of the ^{2}D retention times of all relevant cuts, and has been used to calculate the

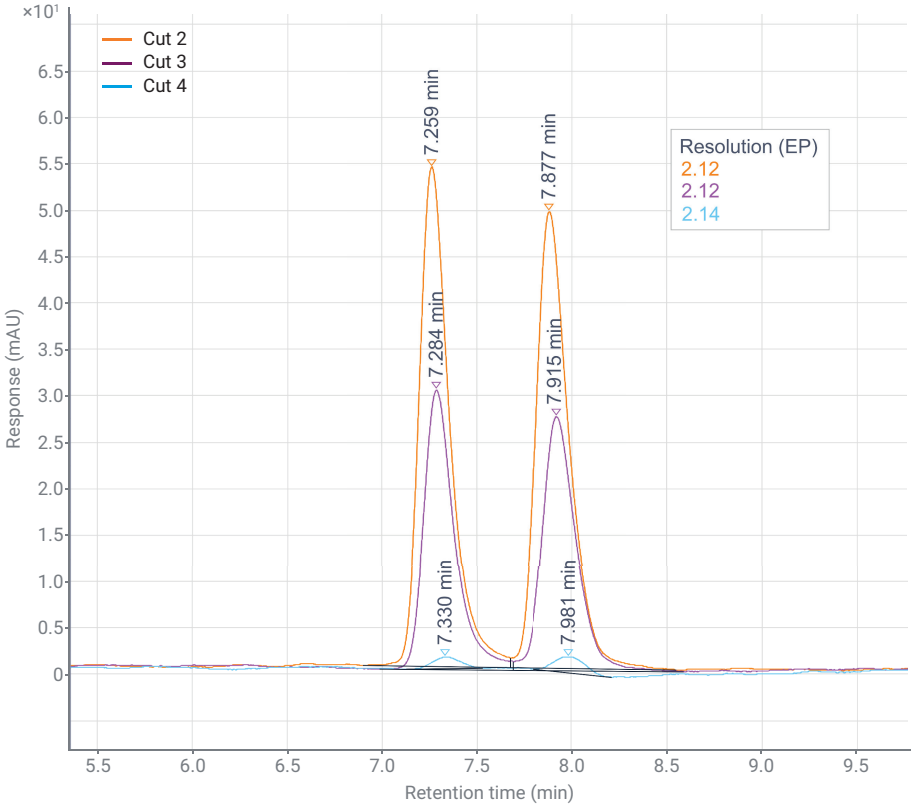

Figure 5. Overlaid ^{2}D chromatograms of cuts 2-4, showing enantioseparation of (S)- and (R)-atenolol contained in Atenolol Impurity Standard obtained from British Pharmacopeia (1 mg/mL).

retention time precision of the (S)- and (R)-atenolol peaks, showing relative standard deviations (RSD) of 0.16 and 0.15% over ten consecutive injections. In the first chromatographic dimension, a retention time precision of 0.03 %RSD was achieved for (R/S)-atenolol using the same dataset. ^{2}D peak areas are calculated as the area sum of all ^{2}D peaks in relevant cuts associated with a ^{2}D compound. Over ten consecutive injections, a ^{2}D peak area precision of 0.19 and 0.25 %RSD could be achieved for (S)- and (R)-atenolol, while (R/S)-atenolol showed a ^{1}D peak area precision of 0.2 %RSD. ^{2}D peak areas were also used to calculate ee, that is theoretically at 0% for racemic mixtures and at 100% for a single, pure enantiomer. The calculated ee ranged from 0.003 to 0.607% over ten consecutive injections, showing the expected values as the sample contained a racemic mixture of the R- and S-enantiomer with a molar ratio of 1:1.

Peak Table ^{2}D

Cut #	^{2}D retention time	Area	Asymmetry 10%	Capacity factor	Plates pm EP	Resol. EP	Selectivity	Signal description	Symmetry	Tailing	Plates EP
Signal: DAD2A, ^{1}D RT: 5.59, ^{2}D RT: 7.27, Compound: (S)-Atenolol, Area: 958.71, Area%: 50											
Signal: DAD2A, ^{1}D RT: 5.59, ^{2}D RT: 7.89, Compound: (R)-Atenolol, Area: 958.64, Area%: 50											
2	7.877	597.133	1.33759	7.29196	105798.58931	2.12076	1.09786	DAD2A Cut 2	0.63803	1.36151	10579.85893
3	7.915	341.862	1.25481	7.33072	100445.27391	2.12117	1.09946	DAD2A Cut 3	0.68615	1.25945	10044.52739
4	7.981	19.644	0.83520	7.40370	95983.79091	2.13967	1.10283	DAD2A Cut 4	0.87129	0.85001	9598.37909

Figure 6. Peak table ^{2}D showing ^{2}D retention time, peak area, and relevant parameters for SST.

Conclusion

Chromatographic separation of synthetic byproducts from APIs, as well as separation of enantiomeric variants of APIs, is an essential part of the drug development process to assure drug safety and potency. This application note describes a 2D-LC workflow for reversed phase separation of (R/S)-atenolol from its impurities in the first chromatographic dimension, and chiral separation of (R)- and (S)-atenolol in the second chromatographic dimension. Agilent 2D-LC Software for OpenLab CDS enabled easy and fast 2D-LC method setup using features such as interactive cut editing and automated stop time calculation. 2D-LC data analysis provided a simple display of data for review and easy access to peak performance parameters for SST. The achiral-chiral 2D-LC separation resulted in excellent reproducibility of peak areas and retention times in both chromatographic dimensions, as well as baseline separation of (R)- and (S)-atenolol.

References

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