Determination of Beta-Blockers in Urine Using Supercritical Fluid Chromatography and Mass Spectrometry

Application Note

Doping Control

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Abstract

This Application Note demonstrates the use of the Agilent 1260 Infinity Analytical SFC System for the separation of a library of polar pharmaceutical compounds. It is focused on the larger number of beta-blockers included in the used library. For all beta-blockers, performance data such as linearity, limit of detection (LOD) and limit of quantification (LOQ), and retention time and area RSDs are discussed. Finally, a urine sample spiked with a beta-blocker was measured.

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Introduction

A subsection of a larger library of polar compounds, listed as prohibited substances by the World Anti-Doping Agency (WADA)[1], has been analyzed under HILIC conditions[2].

In this study, this compound library was investigated by supercritical fluid chromatography (SFC) to evaluate the separation capabilities for such polar compounds and their detection by triple-quadrupole mass spectrometry. Due to the high sample load in doping control analysis, a fast method for the analysis of known doping compounds is required. Due to its fast separation capabilities, SFC can play a significant role to cope with the number of samples in this application area. For the evaluation, the 13 beta-blockers inherent in the used library were used to determine typical limit of detection (LOD), limit of quantification (LOQ), retention time and area RSDs, as well as precision and accuracy data from the quantification of a sample.

Experimental

Instrumentation

Agilent 1260 Infinity Analytical SFC Solution (G4309A):

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High-Performance Degasser
- Agilent 1260 Infinity SFC Standard Autosampler
- Agilent 1260 Infinity Thermostatted Column Compartment
- Agilent 1260 Infinity Diode Array Detector with high-pressure SFC flow cell
- Agilent 6460 Triple Quadrupole LC/MS System (G6460C) with Agilent Jet Stream
- Agilent 1260 Infinity Isocratic Pump (G1310B)
- Agilent Splitter kit G4309-68715

Instrumental setup

Figure 1 shows the recommended configuration of the Agilent 1260 Infinity Analytical SFC Solution with the Agilent 6460 Triple Quadrupole LC/MS System.

Column

Agilent ZORBAX NH2,
4.6 × 150 mm, 5 µm (p/n 883952-708)

Software

- Agilent MassHunter Data Acquisition Software for triple quadrupole mass spectrometer, Version 07.01
- Agilent MassHunter Qualitative Software, Version 07.00
- Agilent MassHunter Quantitative Software, Version 07.00

Connection of the SFC to the MS by splitting and make-up flow

Make up composition

Methanol/Water (95/5), 0.5 mM Ammonium Formate, + 0.2 % formic acid

Make-up flow

0.5 mL/min

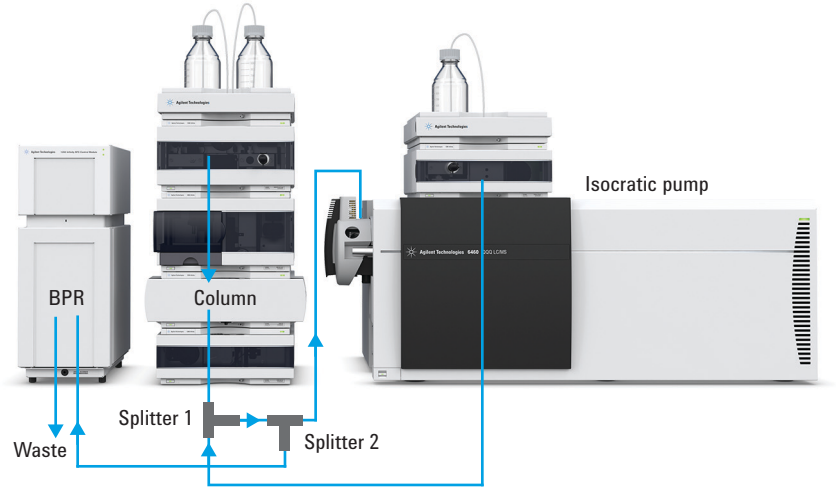


Figure 1. Configuration of the Agilent 1260 Infinity Analytical SFC Solution with the Agilent 6460 Triple Quadrupole LC/MS System. The column is directly connected to splitter 1 in the splitter assembly (BPR = backpressure regulator, UV detector not used, splitter kit p/n G4309-68715).

Standards

The compound library comprised polar pharmaceutical compounds including 13 beta-blockers at a concentration of 1 mg/mL each in acetonitrile. To prepare the stock solution for the described work, a 1:100 dilution in methanol was made.

Chemicals

All chemicals were purchased from Sigma-Aldrich, Taufkirchen, Germany. All solvents were LC/MS grade. Methanol was purchased from J.T. Baker, Germany. 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).

Sample preparation

A urine sample was spiked with penbutolol (250 ng/mL), diluted 1:5 with methanol, vortexed, and centrifuged at 14,000 g for 5 minutes. The supernatant was filtered, and the filtrate was used directly for injection.

SFC method

Parameter	Value
SFC flow	3 mL/min
SFC gradient	0 minutes, 2 %B; 10 minutes, 50 %B
Stop time	10 minutes
Post time	2 minutes
Modifier	Methanol + 0.1 % formic acid (FA)
BPR temperature	60 °C
BPR pressure	150 bar
Column temperature	40 °C
Injection volume	5 µL, three-times loop overfill

MS method

Parameter	Value
Ionization mode	positive
Capillary voltage	2,500 V
Nozzle voltage	2,000 V
Gas flow	8 L/min
Gas temperature	220 °C
Sheath gas flow	12 L/min
Sheath gas temperature	380 °C
Nebulizer pressure	25 psi
MRM conditions	See Table 1, showing detailed retention time, retention time window, and fragmentor and collision energies

Table 1. MRM conditions for the studied beta-blockers, listing precursor ion mass, fragment ion mass, and fragmentor and collision energies.

	Precursor ion (m/z)	Fragmentor (V)	Quantifier	CE (V)	Qualifier	CE (V)
Penbutolol	292	113	236	12	74	20
Alprenolol	250	11	116	16	56	28
Oxprenolol	266	113	116	12	72	16
Bisoprolol	326	141	116	16	74	28
Esmolol	296	121	145	24	56	32
Propranolol	260	95	116	16	56	28
Celiprolol	380	136	251	20	74	32
Acebutolol	337	145	116	20	56	28
Nebivolol	406	151	151	28	44	44
Pindolol	249	110	116	16	56	28
Sotalol	273	83	255	8	133	28
Atenolol	267	110	145	28	56	28
Nadolol	310	88	254	12	56	36

Results and Discussion

The separation of the 13 beta-blockers inherent in the 44-compound drug mix was performed on an amino column with a gradient of methanol (+0.1 %FA) as modifier, starting at 2 % and going up to 50 % in 10 minutes. With this gradient, all 44 compounds could be separated. In this example, only the 13 beta blockers were monitored, and are discussed in detail (Figure 2).

The beta-blockers eluted between 4.68 and 7.38 minutes with a minimum of coelution. The first compound of the whole drug mix eluted at a retention time of 2.27 minutes, with the last one eluting at 8.19 minutes, respectively.

For all beta-blockers, calibration curves were generated from 1,000 ng/mL down to 1 ng/mL (in modifier) to measure linearity, LOQ (S/N > 10), and LOD (S/N > 3) (Table 2). Typical LODs are below 1.5 ng/mL, and typical LOQs are below 5 ng/mL. Linear calibration was obtained for all compounds, with an R^2 better than 0.9990.

The 100 ng/mL calibration concentration was injected 10 times for a statistical evaluation (Table 2). The measured retention time RSDs are typically below 0.25 %, and the area RSDs are below 5 %.

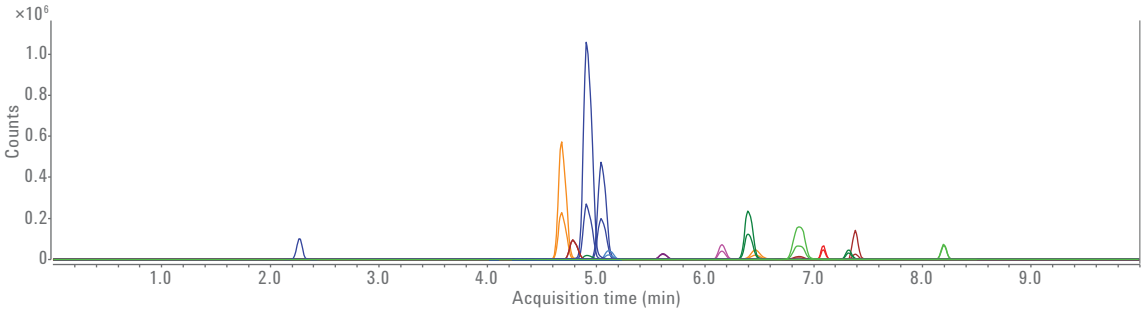
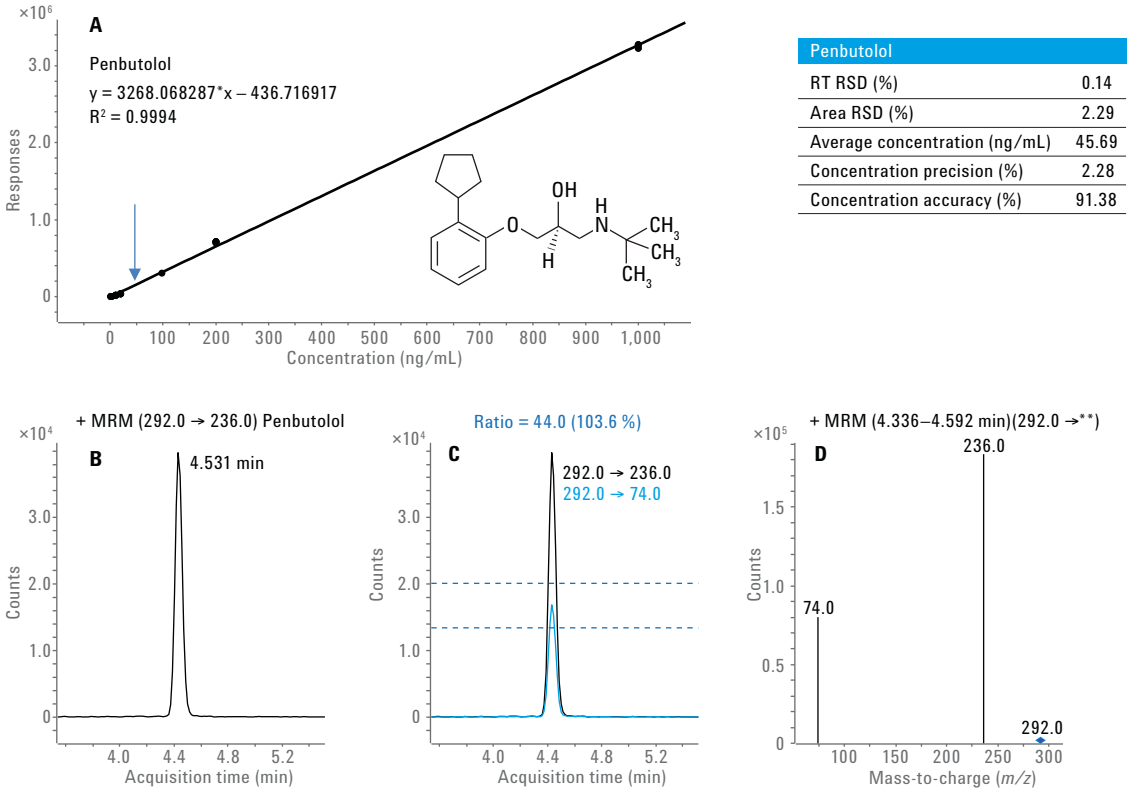


Figure 2. Separation of 13 beta-blockers from a library of 44 compounds. The applied gradient to separate all compounds went from 2 to 50 % methanol in 10 minutes. The earliest eluting compound has a retention time of 2.270 minutes, and the latest eluting compound has a retention time of 8.198 minutes. The beta-blockers eluted between 4.68 and 7.38 minutes.

Table 2. Retention time, RSD's of retention time and areas as well as LODs, LOQs, and linearity of the 13 beta-blockers. The LODs and LOQs are calculated from calibration curves created between 1 and 1,000 ng/mL. The RSDs are calculated from 10 injections of the 100 ng/mL calibration point.

Compound	RT (min)	RT RSD (%)	Area RSD (%)	LOD (ng/mL)	LOQ (ng/mL)	R^2
Penbutolol	4.680	0.18	3.54	0.20	1.60	0.9994
Alprenolol	4.784	0.16	4.42	1.50	5.00	0.9990
Oxprenolol	4.912	0.16	2.76	0.10	0.25	0.9992
Bisoprolol	5.045	0.15	3.00	0.19	0.60	0.9991
Esmolol	5.111	0.15	6.83	1.41	4.71	0.9992
Propranolol	5.611	0.18	8.05	1.71	5.70	0.9997
Celiprolol	6.158	0.26	3.29	0.37	1.25	0.9998
Acebutolol	6.397	0.26	1.68	0.66	2.19	0.9997
Nebivolol	6.463	0.24	5.31	2.85	9.52	0.9996
Pindolol	6.854	0.28	3.67	3.64	10.90	0.9994
Sotalol	7.088	0.24	6.29	5.51	18.38	0.9995
Atenolol	7.322	0.28	3.73	1.98	6.66	0.9995
Nadolol	7.383	0.29	2.53	0.41	1.38	0.9997

As an example of a real-life sample, penbutolol was spiked into urine at a concentration of 250 ng/mL, and prepared as described in the experimental section. Because pure aqueous samples should not be injected into the SFC, the sample was diluted 1:5 in methanol. For the quantitative determination of the penbutolol, a calibration curve from 1,000 ng/mL down to 2 ng/mL was created in solvent (Figure 3A). The measured average concentration of the diluted sample was 45.59 ng/mL, and the determined sample concentration 228.45 ng/mL. The compound eluted at 4.53 minutes with good signal intensity (Figure 3B) and the quantifier/quantifier ratio was in the expected range (Figure 3C). For a statistical evaluation, the sample was injected 10 times, and the retention time and area RSD were calculated to be 0.14 and 2.29 %, respectively. The concentration precision and the accuracy was calculated to be 2.28 and 91.38 %, respectively.



Penbutolol	
RT RSD (%)	0.14
Area RSD (%)	2.29
Average concentration (ng/mL)	45.69
Concentration precision (%)	2.28
Concentration accuracy (%)	91.38

Figure 3. Determination of penbutolol in urine. A) Calibration curve of penbutolol between 2 and 1,000 ng/mL. The concentration measured in the diluted sample is indicated by the arrow. B) Quantifier transition of penbutolol from the diluted sample. C) Quantifier/qualifier ratio of the measured sample. D) MRM transitions measured for penbutolol in the sample. The table inset shows retention time RSD and area RSD as well as average concentration, concentration precision and concentration accuracy calculated from 10 injections of the sample.

Conclusion

This Application Note demonstrates the possibility to separate a large number of highly polar pharmaceutical compounds within a short run time by SFC. Their detection by connecting the SFC to a triple-quadruple mass spectrometer with limits of detection typically below 1.5 ng/mL has been shown. The retention time RSDs were below 0.25 %, and area RSDs were below 5 %.

It was demonstrated that pharmaceutical compounds could be measured in an aqueous real-life sample by dilution with organic solvents with sufficient sensitivity and concentration precision and accuracy.

References

1. The World Anti-Doping Code, The 2015 Prohibited List International Standard, World Anti-Doping Agency, Montreal, Canada, 20 September 2014, Available at www.wada-ama.org (accessed November 1, 2015)

2. Mazzarino, M.; *et al.* Screening and confirmation analysis of stimulants, narcotics and beta-adrenic agents in human urine by hydrophobic interaction liquid chromatography coupled to mass spectrometry. *Journal of Chromatography A* **2011**, *1218*, 8156–8167

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