Application Note

Pharma & Biopharma

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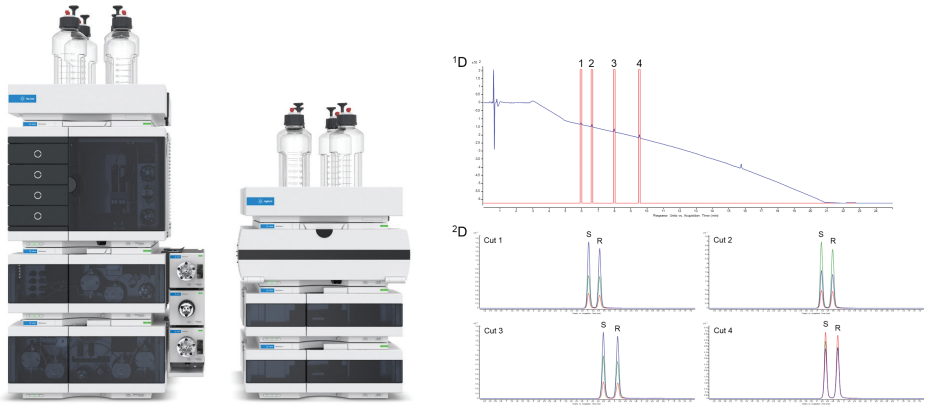
Quantitation of the Chiral Shift in Metabolism of Propranolol Using 2D-LC/MS/MS

The Agilent InfinityLab 2D-LC Solutions for achiral-chiral analysis of complex samples

Suitable for Agilent
1290 Infinity III LC



Authors

Lukas C. Harps,
Felix Bredendiek, and
Maria K. Parr
Freie Universitaet Berlin
Berlin, Germany

Sonja Schipperges,
Bernhard Wuest, and
Andreas Borowiak
Agilent Technologies, Inc.

Abstract

Chiral recognition of drugs and their metabolites plays an important role in comprehension of drug metabolism. Combining achiral and chiral discrimination in one LC separation is challenging for complex biological samples. Two-dimensional liquid chromatography (2D-LC) with a chiral dimension allows the chiral discrimination of analytes in complex biological samples. In this application note, 2D-LC/MS/MS is used to quantitatively investigate chiral shift in the metabolism of propranolol and its hydroxy metabolites in human urine. The online 2D-LC analysis saves time and minimizes sample handling. Analysis of human urine samples after administration of racemic propranolol reveals considerable chiral shifts in propranolol as well as its hydroxy metabolites. Excretion rates of the individual (R)- and (S)-enantiomers are monitored for investigation of the enantioselective metabolism of propranolol.

Introduction

The enantiomers of chiral, biologically active compounds often show differences in pharmacokinetic behavior and pharmacological activity. For this reason, each enantiomer of chiral pharmaceuticals should be considered as single active compound, according to guidelines published by the FDA and EMA.[1,2] Chiral recognition of drugs and their metabolites plays an important role in comprehension of pharmacodynamic effects and pharmacokinetic behavior of chiral drugs.

Two-dimensional liquid chromatography (2D-LC) with a chiral dimension enables the enantiomeric discrimination of analytes in complex biological samples.[3,4]

This application note demonstrates the quantitative investigation of chiral shifts in the metabolism of propranolol (PL) and its hydroxy metabolites in human urine. 2D-LC/MS/MS is used to employ a combination of achiral and chiral separation.[5] In an online 2D-LC analysis, this avoids the need for achiral analysis with fraction collection and fraction reanalysis with a chiral separation. The online 2D-LC analysis saves time and minimizes sample handling.

Experimental

Equipment

The Agilent 1290 Infinity II 2D-LC System comprises the following modules:

- Two Agilent 1290 Infinity II High Speed Pumps (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler (option 100)
- Two Agilent 1290 Infinity II Multicolumn Thermostats (G7116B)
- Two Agilent 1290 Infinity II Diode Array Detectors (G7117B) with Max-Light Cartridge Cell 10 mm (G4212-60008)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2D-LC Valve, active solvent modulation (G4243A)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with multiple heart-cutting valves (G4242-64000) equipped with 40 µL loops

Mass spectrometric detection was performed using an Agilent 6495 triple quadrupole LC/MS system equipped with an Agilent Jet Stream ESI source.

Software

- Agilent OpenLab CDS ChemStation Edition Rev. C.01.08 [210] with 2D-LC Software version A.01.04 SR1.
- ²D Chromatogram Creator for MassHunter Rev. 1.20.
- Agilent MassHunter workstation software LC/MS Data Acquisition Version B.08.02 for 6400 Series triple quadrupole system
- Agilent MassHunter workstation software Quantitative Analysis Version B.09.00

Columns

- Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 2.7 µm (p/n 695775-912)
- Agilent InfinityLab Poroshell 120 Chiral-T, 4.6 × 100 mm, 2.7 µm (p/n 685975-603)

Chemicals

All solvents were LC grade. Methanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Ultrapure Lab Water System equipped with a Millipak 0.22 µm membrane point-of-use cartridge (Millipore, Merck (Darmstadt, Germany)). Ammonium formate and formic acid were purchased from VWR (Darmstadt, Germany) and Sigma-Aldrich (Steinheim, Germany), respectively.

(R/S)-Propranolol (PL)·HCl was purchased from Fluka (Buchs, Switzerland), (R/S)-4'-hydroxy propranolol (4-HOPL)·HCl was obtained from Sigma Aldrich (Taufkirchen, Germany) and (R)-PL·HCl and (S)-4-HOPL·HCl were purchased from Santa Cruz Biotechnologies Inc. (Heidelberg, Germany). (R/S)-5'-hydroxy propranolol (5-HOPL) and (R/S)-7'-hydroxy propranolol (7-HOPL) were synthesized in-house. Chemicals and materials used in the chemical synthesis were obtained from Alfa Aesar, Arcos Organics (Schwerte, Germany), and Sigma-Aldrich. Recombinant human CYP2D6 and CYP1A2, as well as NADPH generating system were purchased from Corning GmbH (Amsterdam, Netherlands).

Calibration standards

Calibration was performed employing matrix-matched calibration standards in a concentration range of 0.2 to 2,000 ng/mL per racemate (equal mixture of both enantiomers). For preparation of the matrix matched calibration standards, racemic stock solutions in methanol were diluted to a concentration of 0.1, 0.5, 1, 5, 10, 50, 100, 500 and 1,000 ng/mL of each enantiomer, with a 1/4 (v/v) mixture of blank urine and methanol, centrifuged with 6,484 g for 10 minutes.

Urine sampling and sample preparation

After a single oral dose of 40 mg of racemic propranolol, postadministration urines were collected for seven days. Urine samples were collected continuously within the first 24 hours. After that, only morning urine samples were collected. Samples were stored at –20 °C until analysis. For analysis, 200 µL of urine were diluted with 800 µL of methanol, and centrifuged with 6,484 g for 10 minutes.

Biosynthesis of (R)-hydroxy propranolol metabolites

Biological syntheses of (R)-4-HOPL, (R)-5-HOPL, (R)-7-HOPL from (R)-PL were performed using isolated recombinant human enzymes, CYP1A2 and CYP2D6.

Results and discussion

According to literature, propranolol (PL) undergoes extensive enantioselective phase-1 and phase-2 metabolism. Cytochrome P450 enzymes (CYP) isoform 2D6, CYP1A2, and CYP2C19 catalyze ring oxidation, side chain dealkylation, and further side chain oxidation of PL.[6] In humans, 4'-hydroxy propranolol (4-HOPL), 5'-hydroxy propranolol (5-HOPL), 7'-hydroxy propranolol (7-HOPL), N-desisopropyl propranolol (NDP), and 3-(1-naphtoxy) lactic acid (NLA) are reported phase-1 metabolites.[7,8] Due to its preference in human phase-1 metabolism,[9] (R)-PL exhibits a shorter half-life.[7]

Separation of PL, 4-HOPL, 5-HOPL, and 7-HOPL and their respective enantiomers, was achieved in one chromatographic run. The employed 2D-LC method combined an achiral separation on a Poroshell 120 Phenyl-Hexyl column in the first dimension (^{1}D), with a chiral separation on a Poroshell 120 Chiral-T column in the second dimension (^{2}D). The ^{1}D peaks of PL, 4-HOPL, 5-HOPL, and 7-HOPL were transferred to the ^{2}D chiral separation in individual heart-cuts. In the ^{2}D chiral separation, enantiomers were baseline-separated with resolutions ranging from 2.4 to 3.1. Figures 1 and 2 show the 2D-LC analysis of PL, 4-HOPL, 5-HOPL, and 7-HOPL with UV and MS detection.

Method

First Dimension (^{1}D)		
Column	Agilent Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 2.7 µm	
Solvent	A) 10 mM ammonium formate in water, adjusted to pH 3 B) Methanol	
Gradient	0 minutes – 5% B 2 minutes – 5% B 4 minutes – 30% B 20 minutes – 95% B Stop time: 25 minutes Post-time: 5 minutes	
Injection	Injection volume: 2 µL; 3 seconds needle wash in water/acetonitrile (50/50; v/v)	
Flow rate	0.400 mL/min	
Temperature	30 °C	
Detection	UV	
UV	230/4 nm and 290/4nm, reference 370/60 nm, 20 Hz	
Second Dimension (^{2}D)		
Column	Agilent Poroshell 120 Chiral-T, 4.6 × 100 mm, 2.7 µm	
Solvent	A) 10 mM ammonium formate in methanol B) 10 mM ammonium formate in methanol	
Temperature	25 °C	
Detection	UV and MS	
UV	230/4 nm and 290/4nm, reference 370/60 nm, 20 Hz	
Triple Quadrupole LC/MS	Spray Chamber	Agilent Jet Stream Electrospray
	Scan Type	MRM
	Source	
	Drying Gas Temperature	160 °C
	Drying Gas Flow	11 L/min
	Nebulizer Pressure	30 psi
	Sheath Gas Temperature	375 °C
	Sheath Gas Flow	12 L/min
	Capillary Voltage	3,000 V
	VCharging	500 V
	Ion Funnel	
	Pos high pressure RF	150
	Pos low pressure RF	60
	MRM Transitions	

Compound	Precursor Ion	Product Ion	CE	Polarity
Hydroxy propranolol	276.2	116.1	16	Positive
Hydroxy propranolol	276.2	72.0	12	Positive
Hydroxy propranolol	276.2	58.0	44	Positive
Propranolol	260.2	183.1	20	Positive
Propranolol	260.2	116.1	20	Positive
Propranolol	260.2	56.0	36	Positive

The following settings were used for all scan segments:
MS1 res: Unit; MS2 res: Unit; Dwell: 60; Frag: 380 V; Cell acc: 4 V

2D-LC	
Configuration	2D-LC valve ASM + 2 MHC valves; 40 µL loops; countercurrent
2D-LC mode	Heart-cutting
Gradient	50% B, isocratic ^{2}D gradient stop time: 7.95 minutes ^{2}D cycle time: 8.00 minutes
Flow Rate	1.000 mL/min
Sampling Table	The cut times in the sampling table were set up according to the ^{1}D retention times of the target peaks: 5.90 minutes, time-based 6.56 minutes, time-based 7.93 minutes, time-based 9.47 minutes, time-based

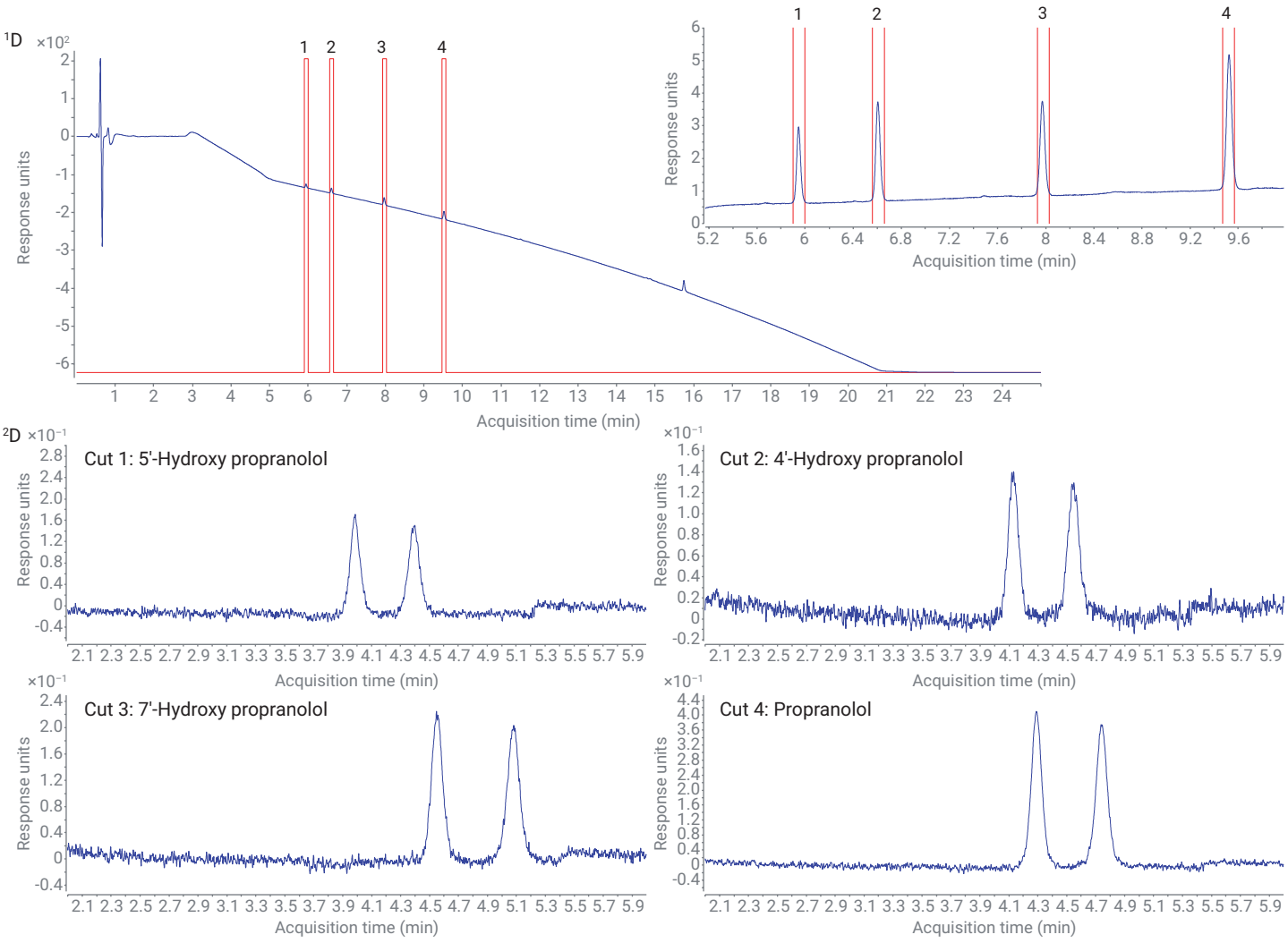


Figure 1. UV chromatograms showing 2D-LC analysis of a 1,000 ng/mL mixture of PL, 4-HOPL, 5-HOPL, and 7-HOPL. Top: ^{1}D chromatogram acquired at 230 nm (inset acquired at 290 nm) with marked heart-cuts. Bottom: ^{2}D chromatograms acquired at 290 nm.

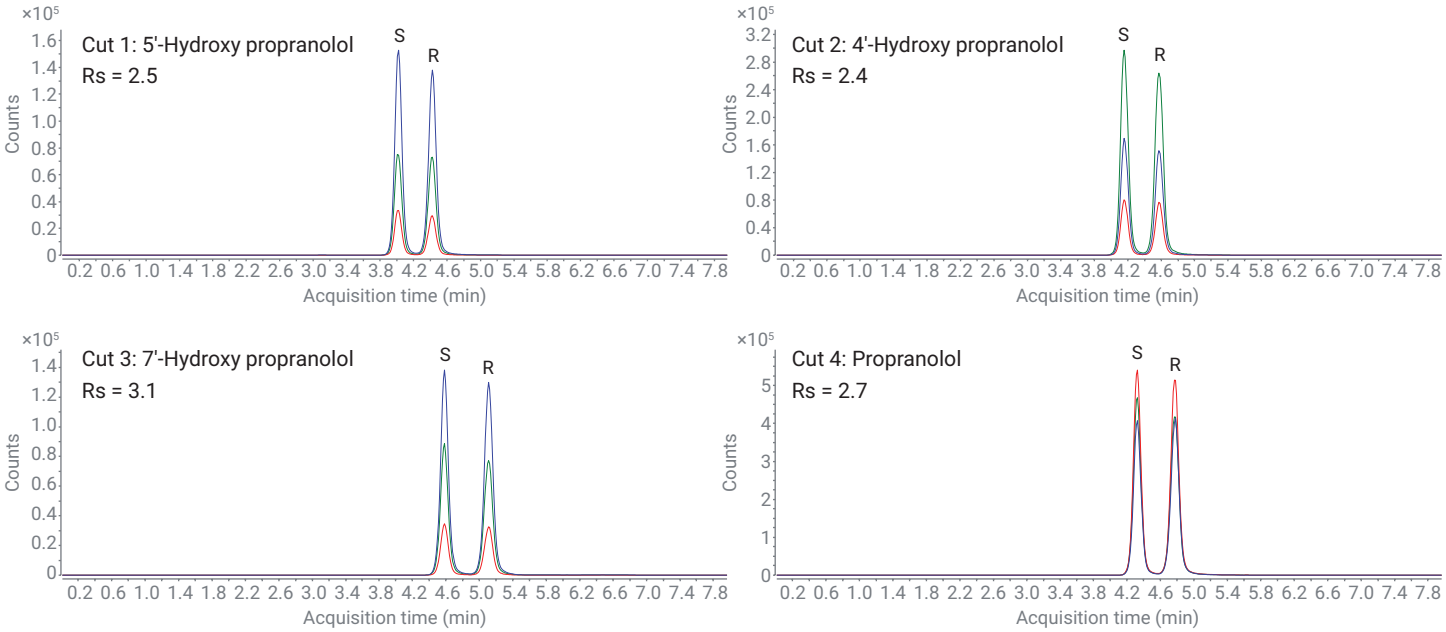


Figure 2. [2]D MS chromatograms displaying 2D-LC analysis of a 1,000 ng/mL mixture of PL, 4-HOPL, 5-HOPL, and 7-HOPL. MRM transitions: Hydroxy propranolols: 276.2 → 116.1 (blue), 276.2 → 72.0 (red), 276.2 → 58.0 (green); propranolol: 260.2 → 183.1 (blue), 260.2 → 116.1 (red), 260.2 → 56.0 (green).

Allocation of enantiomeric elution orders was achieved using (R)-PL and (S)-4-HOPL as references. Additionally, (R)-PL was incubated *in vitro* with isolated human recombinant CYP1A2 and CYP2D6. In this manner, biosynthesized (R)-PL metabolites, (R)-4-HOPL, (R)-5-HOPL, and (R)-7-HOPL, were obtained as references. Figures 3 and 4 show the [2]D MS chromatograms of samples obtained from incubation of (R)-PL with CYP1A2 and CYP2D6, respectively. Considering the retention times observed in the analysis of a racemic mixture of PL and its hydroxy metabolites (see Figure 2), we can conclude that (R)-enantiomers of ring hydroxylated PL, and of PL itself, are more retained in the chiral [2]D separation than their (S)-enantiomers.

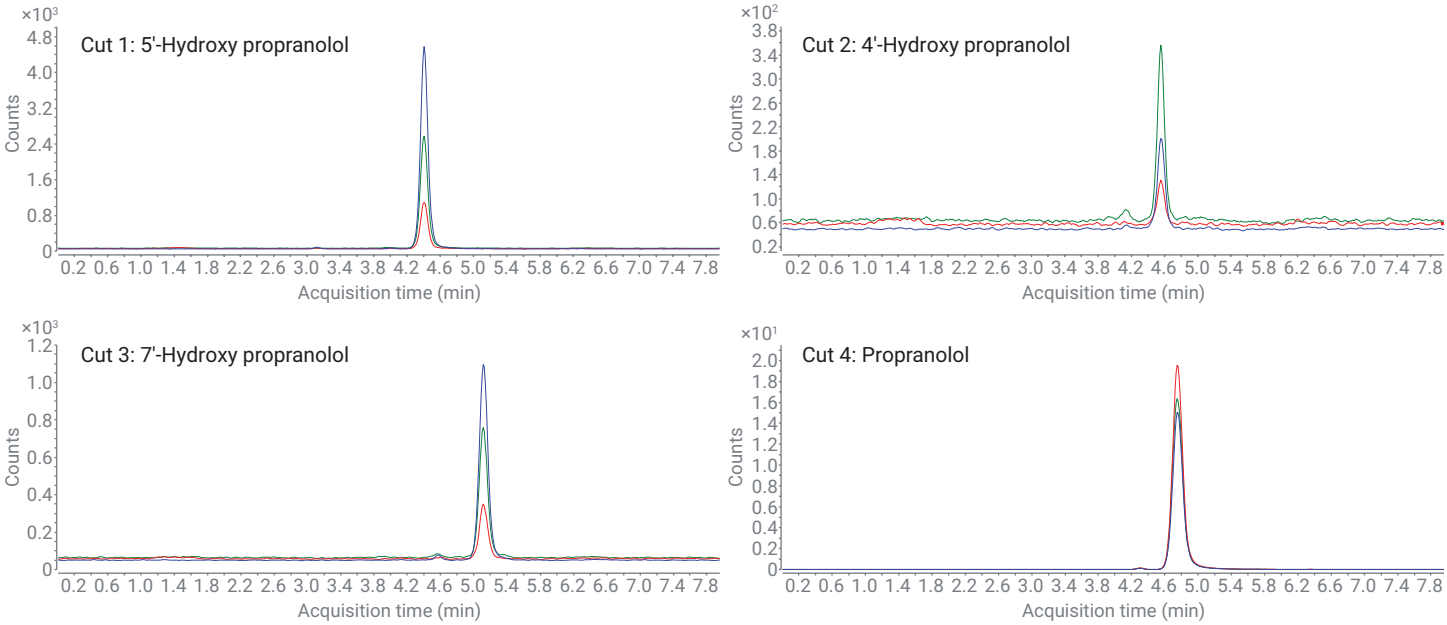


Figure 3. [2]D MS chromatograms of 2D-LC analysis of (R)-PL incubated *in vitro* with isolated human recombinant CYP1A2. MRM transitions: Hydroxy propranolols: 276.2 → 116.1 (blue), 276.2 → 72.0 (red), 276.2 → 58.0 (green); propranolol: 260.2 → 183.1 (blue), 260.2 → 116.1 (red), 260.2 → 56.0 (green).

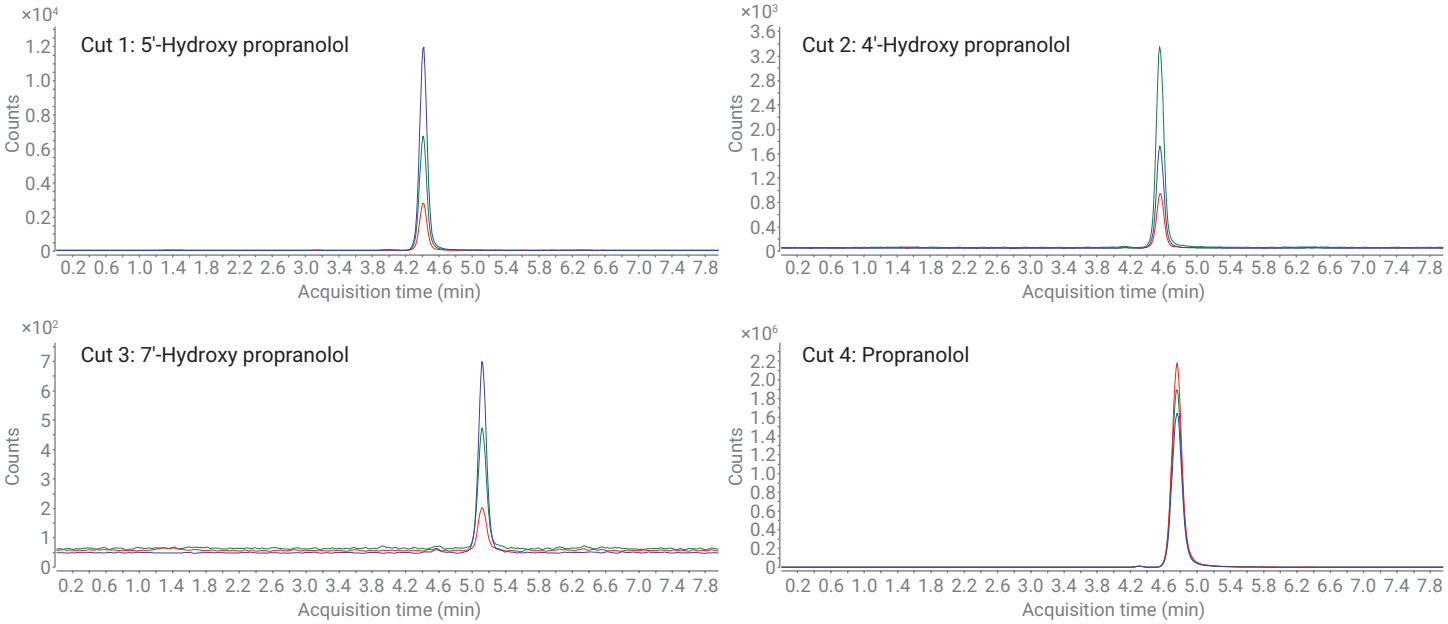


Figure 4. [2]D MS chromatograms of 2D-LC analysis of (R)-PL incubated *in vitro* with isolated human recombinant CYP2D6. MRM transitions: Hydroxy propranolols: 276.2 → 116.1 (blue), 276.2 → 72.0 (red), 276.2 → 58.0 (green); propranolol: 260.2 → 183.1 (blue), 260.2 → 116.1 (red), 260.2 → 56.0 (green).

The 2D-LC method was characterized in terms of retention time precision, calibration, limit of detection (LOD), and limit of quantification (LOQ). The concentrations of PL, 4-HOPL, 5-HOPL, and 7-HOPL in urine samples are too low to allow UV detection. For this reason, time-based heart-cutting is employed. [1]D retention time stability is crucial for reliable transfer of PL, 4-HOPL, 5-HOPL, and 7-HOPL from the [1]D to the [2]D. [1]D retention time precision was investigated from matrix matched calibration standards at 500 and 1,000 ng/mL. The 1,000 ng/mL standard was also analyzed as a control sample to confirm [1]D retention time stability in between sample analyses. Retention time precision was excellent, allowing reliable transfer of PL, 4-HOPL, 5-HOPL, and 7-HOPL from the [1]D to the [2]D (see Table 1).

Table 1. [1]D retention time precision determined from the analysis of matrix matched calibration standards at 500 and 1,000 ng/mL (n = 6).

Compound	RT (min)	RT RSD (%)
4'-Hydroxy Propranolol	6.61	0.11
5'-Hydroxy Propranolol	5.95	0.10
7'-Hydroxy Propranolol	7.98	0.10
Propranolol	9.53	0.07

Matrix effects were investigated from the analysis of calibration standards in neat solvent and matrix matched calibration standards. Since matrix effects were observed, matrix matched calibration was carried out.

Regression type of calibrations were proven using the Mandel test (with P = 0.99). Considering the concentration range used for quantification (0.1 to 1000 ng/mL), the Mandel test showed that quadratic regression was appropriate for the individual (R)- and (S)-enantiomers of PL, 4-HOPL, 5-HOPL, and 7-HOPL. Also, weighted $1/x^2$ calibration was used, according to Gu *et al.*[10]

LOD and LOQ were calculated using a calibration curve-based method. Calibration standards in the range of 0.1 to 5 ng/mL were chosen for determination of LOD and LOQ. In this concentration range, a linear correlation was found suitable using the Mandel test.

Correlation of calibration curves in the concentration range of 0.1 to 1,000 ng/mL, as well as LOD and LOQ, are shown in Table 2. Accuracy was determined to be between 80 and 120% using matrix-matched calibration.

Analysis of human urine samples after administration of a single oral dose of 40 mg racemic propranolol revealed considerable chiral shifts in PL and its hydroxy metabolites. This is clear from the differences in the enantiomeric excess (S/R ratio) observed for PL, 4-HOPL, 5-HOPL, and 7-HOPL during the analysis of the urine sample after 5.1 hours. This is shown in Figure 5.

Urinary excretion rates of the individual (R)- and (S)-enantiomers of PL and its hydroxy metabolites were calculated using the determined concentrations in urine samples. Enantiomeric excess was calculated based on peak areas. The (R)- and (S)-enantiomers of PL showed maximum excretion rates of 281 ng/min and 237 ng/min, respectively. These maximum excretion rates occurred at 7.3 hours, representing resorption, distribution, and first pass effect. (R)-PL showed slightly higher excretion rates across all time points. Compared to the excretion rates of PL, the excretion rates of its hydroxy metabolites were factors of 10 to 100 lower. Maximum excretion rates of the hydroxy metabolites occurred no later than 5.1 hours after administration of racemic PL. The S/R excretion ratio of 4-HOPL seemed to follow the substrate's S/R excretion ratio. The S/R excretion ratio of 5-HOPL and 7-HOPL, in contrast, were below 0.13 across all time points. A detailed discussion of the observed urinary excretion rates and the enantiomeric excess of propranolol and its hydroxy metabolites can be found elsewhere.[5]

Table 2. Correlation of calibration curves, LOD, and LOQ.

Compound	R^2	LOD (pg/mL)	LOQ (pg/mL)
(R)-4'-Hydroxy Propranolol	0.9838	8.6	50.4
(S)-4'-Hydroxy Propranolol	0.9848	8.3	48.7
(R)-5'-Hydroxy Propranolol	0.9881	46.7	265.1
(S)-5'-Hydroxy Propranolol	0.9887	36.2	206.8
(R)-7'-Hydroxy Propranolol	0.9735	37.7	217.2
(S)-7'-Hydroxy Propranolol	0.9746	45.0	257.7
(R)-Propranolol	0.9934	18.4	107.3
(S)-Propranolol	0.9939	39.7	228.0

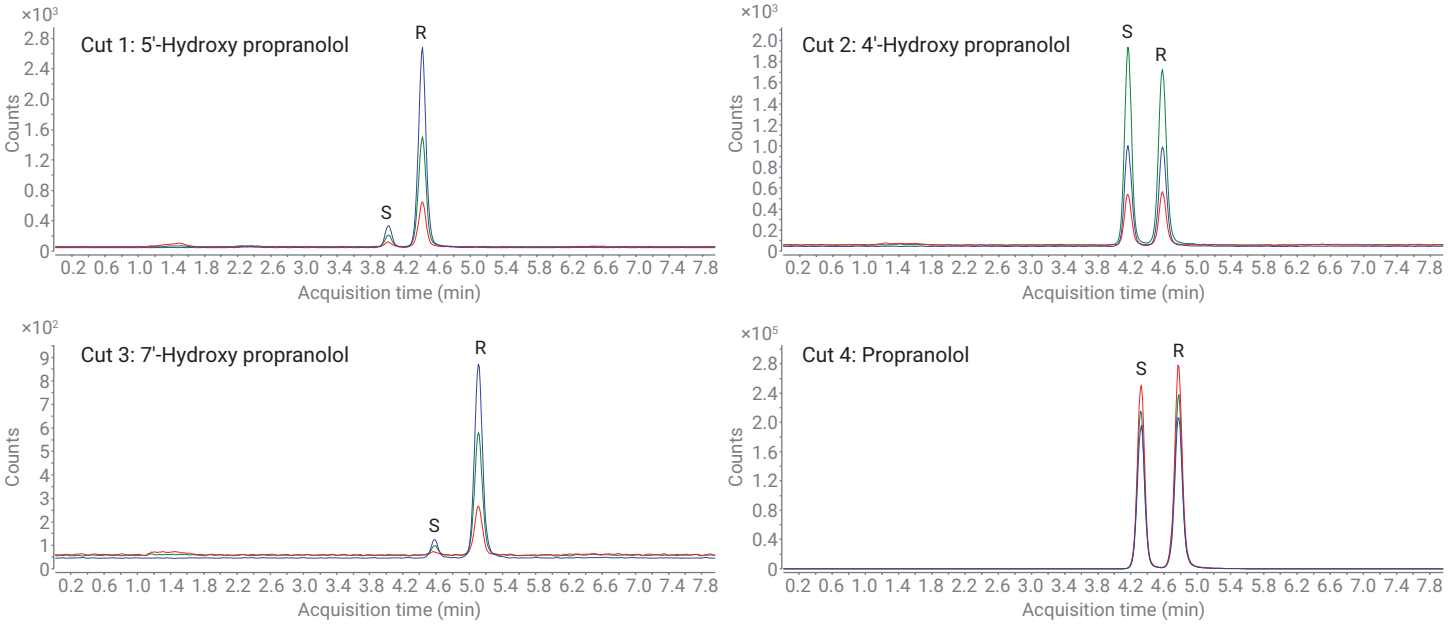


Figure 5. [2]D MS chromatograms of 2D-LC analysis of urine sample after 5.1 hours. MRM transitions: Hydroxy propranolols: 276.2 → 116.1 (blue), 276.2 → 72.0 (red), 276.2 → 58.0 (green); propranolol: 260.2 → 183.1 (blue), 260.2 → 116.1 (red), 260.2 → 56.0 (green).

Conclusion

Two-dimensional liquid chromatography (2D-LC) with a chiral dimension facilitates the enantiomeric discrimination of analytes in complex biological samples. Compared to an offline approach, the online 2D-LC analysis saves time and minimizes sample handling.

The 2D-LC analysis with a chiral dimension was used to quantitatively investigate the chiral shift in metabolism of propranolol and its hydroxy metabolites in human urine. After administration of racemic propranolol, considerable chiral shifts in propranolol and its hydroxy metabolites 4'-hydroxy propranolol, 5'-hydroxy propranolol, and 7'-hydroxy propranolol, could be observed. Excretion rates of the individual (R)- and (S)-enantiomers were monitored for investigation of the enantioselective metabolism of propranolol.

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