

Application of column-switching with ultra high pressure liquid chromatography for the quantitative analysis of pharmaceuticals in plasma

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Overview

Investigation of column-switching in quantitative ultra high pressure liquid chromatography (UHPLC) analysis.

- 1) Comparison of direct injection vs. column-switching (sample trapping).
- 2) Evaluation of dwell times in SRM mode for UHPLC-MS/MS.
- 3) Validation of a buprenorphine assay in human plasma by LC-MS/MS.
- 4) Comparison of quantitative performances between UHPLC-MS/MS and regular LC-MS/MS with column-switching setup.

Introduction

Quantitative analysis of pharmaceutical compounds in biological fluids is mainly performed by liquid chromatography and mass spectrometric detection. Matrix effects and possible interferences with metabolites require good chromatographic separations which often reduces sample throughput.

The application of sub 2 µm particle allows to improve the separation power and to reduce the analysis time. However, injection of large sample volumes jeopardizes the system performance. Large sample volumes can be injected onto chromatographic columns with reduced column volume using column-switching approaches without compromising separation efficiency.

The aim of the present work was to compare the performance of a LC-MS/MS assay using various HPLC particle size (1.9 and 3 µm) for the quantitation of buprenorphine and naltrexone in human plasma.

Discussion

Small particle size UHPLC columns allow to inject limited sample volume. A volume up to 10 µl could be injected directly onto the column without compromising chromatographic performance.

With the column-switching setup, up to 5 times larger sample volume (i.e. 50 µl) could be injected.

With chromatographic peak widths of 3-6 seconds, the SRM transition dwell times have to be adapted (i.e. faster cycle time) in order to acquire enough data points within each peak for adequate quantitation.

For the buprenorphine assay in human plasma, liquid-liquid extraction was selected in order to avoid UHPLC column clogging when performing backflushed column-switching experiments.

With the UHPLC method, the analysis time could be improved by a factor of 2.5 without losing chromatographic resolution.

The use of ultra high pressure column-switching UHPLC-MS/MS did improve the quantitative performances in the low end concentration of the assay when compared to the regular LC-MS/MS assay (i.e. sensitivity, precision and accuracy).

Finally it's noteworthy that the trapping column, which is not meant to support ultra high pressure, is generating some peak broadening and prevents to fully benefit from the UHPLC performance in terms of sensitivity increase.

Methods

Plasma samples

Human blood samples collected at the Geneva University Hospital, pooled with citrate anti-coagulant and stored at -20°C before use. Spiked with concentrated aqueous/organic solutions of standard compounds (i.e. 2% v/v of plasma volume).

Liquid-liquid extraction (LLE)

To 200 µl of fortified plasma sample were added 20 µl of internal standard (IS), 10 µl of 10% aq. triethylamine and 800 µl of n-butyl chloride:acetonitrile (4:1, v/v) solution and vortex-mixed.

The organic phase was transferred to clean tubes and evaporated to dryness after centrifugation (14'000 x g, 10 min. at 20°C). Extracted samples were reconstituted with 100 µl of 1% formic acid in water:methanol (9:1, v/v) solution.

Instrumentation (LC and UHPLC)

Autosampler | HTS PAL (CTC Analytics, Switzerland) equipped with a 6-ports high pressure injection valve (Rheodyne, USA) and a 10-ports high pressure column-switching valve (VICI, Switzerland).

Loading pump | LC-10ATvp (Shimadzu, Japan) low pressure quaternary gradient pump (FCV-10ALvp) with a SCL-10Ayp controller.

Trap column | Kromasil C18 (5 µm) - 8 mm x 3 mm I.D. (Macherey-Nagel, Switzerland)
conditions | 0.1% aq. TFA - 1 ml/min.

UHPLC Pump | Rheos Allegro UHPLC (Thermo Scientific, Switzerland) low pressure quaternary gradient pump.

LC column | Polaris C18 (3 µm) - 50 mm x 2.0 mm I.D. (MetaChem Technologies, USA)
conditions | A = 1% FA in water:methanol (95:5, v/v)
B = 1% FA in water:methanol (5:95, v/v)
hold 0.4 min. at initial conditions after switching
0 - 50% B in 0.5 min. - hold 0.5 min.
50 - 90% B in 2.0 min. - hold 1.0 min. (wash)
flow rate = 250 µl/min. - total time = 8.0 min.

UHPLC column | Hypersil GOLD (1.9 µm) - 50 mm x 2.1 mm I.D. (Thermo Scientific, UK)
conditions | A,B = same as LC conditions
hold 0.2 min. at initial conditions after switching
0 - 50% B in 0.2 min. - hold 0.2 min.
50 - 90% B in 0.8 min. - hold 0.4 min. (wash)
flow rate = 600 µl/min. - total time = 3.4 min.

Mass Spectrometry (MS)

4000 Q TRAP (Applied Biosystems | MDS Sciex, Canada). MS/MS spectra were acquired in SRM mode with MS compound-dependent parameters optimized on standard solutions.

conditions | IS = 5'200 V
Neb. gas = 50 - Turbo gas = 50 - T = 650 °C
Curtain gas = 10 - CAD gas = 4

SRM transitions
buprenorphine m/z 468.4 - m/z 55.0
buprenorphine-d4 m/z 472.4 - m/z 59.0 (IS 1)
naltrexone m/z 342.3 - m/z 55.0
6-beta naltrexol m/z 344.4 - m/z 55.0
naltrexone m/z 328.4 - m/z 51.2 (IS 2)

Results

1 Optimisation of column-switching conditions in UHPLC-MS/MS

Loading capacity of UHPLC columns is somehow limited due to the small particles size of their packing material. Therefore, increasing volumes of a standard solution were injected either directly onto the Hypersil GOLD column (Fig. 1), or via the column-switching setup (Fig. 2).

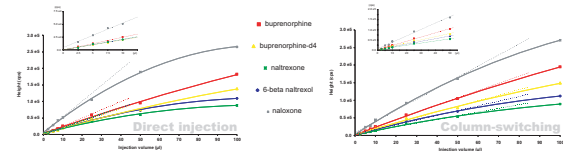


Fig. 1 | Direct injection of increasing volumes of a standard solution at 0.025 ng/ml by UHPLC-MS/MS.

Different loading time (i.e. from 3 to 30 sec.) were evaluated for each injected volume in order to check that no loss of sample occurred by elution from the trapping column. With a loading phase flow rate of 1 ml/min, 6 seconds were sufficient to load up to 100 µl of plasma sample onto the C18 trap column. Linearity of analyte's response was observed up to 10 µl injected in direct injection (inset Fig. 1), and up to 50 µl with column-switching (inset Fig. 2) without the loss of chromatographic performances.

3 Validation of a buprenorphine assay in plasma by LC-MS/MS

The buprenorphine assay was validated from 0.10 to 100 ng/ml (n=5) in plasma with regular column-switching LC-MS/MS conditions.

Expected Concentration (ng/ml)	Sample Name	Mean (ng/ml)	Standard Deviation	%CV	Accuracy (%)
0.10	CO3	0.10	0.00	1.55	99.3
0.25	CO4	0.26	0.02	6.35	104
0.50	CO5	0.47	0.05	11.4	93.9
1.00	CO6	1.04	0.03	2.42	104
2.50	CO7	2.49	0.08	3.33	99.5
5.00	CO8	5.04	0.23	4.57	104
25.0	CO9	24.8	0.36	1.43	99.3
50.0	CO10	47.3	1.36	2.88	94.6
100	CO11	101	2.98	2.96	101

Expected Concentration (ng/ml)	Sample Name	Mean (ng/ml)	Standard Deviation	%CV	Accuracy (%)
0.10	QC3	0.11	0.02	13.9	114
0.25	QC4	0.26	0.03	10.2	100
0.50	QC5	0.51	0.02	4.76	100
1.00	QC6	1.07	0.04	3.88	107
2.50	QC7	2.60	0.13	4.88	104
5.00	QC22	5.22	0.17	3.22	104
10.0	QC8	10.8	0.74	6.97	108
25.0	QC3	25.2	0.78	3.10	101
50.0	QC10	47.8	1.16	2.42	95.9
100	QC11	106	6.86	6.27	106

Naltrexone and its metabolite 6-beta naltrexol were validated from 0.05 to 2.50 ng/ml (n=5) in plasma.

2 Evaluation of SRM dwell time in UHPLC-MS/MS

Different dwell times were evaluated for the SRM transitions in order to have sufficient data points to characterize each chromatographic peaks. Figure 3 shows extracted ion chromatograms of the buprenorphine transition when a standard solution of 0.5 ng/ml is analyzed by column-switching UHPLC-MS/MS.

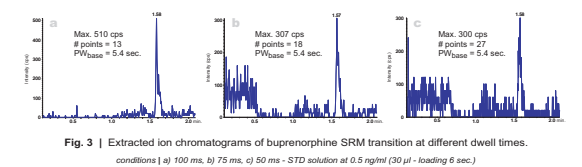


Fig. 3 | Extracted ion chromatograms of buprenorphine SRM transition at different dwell times. conditions | a) 100 ms. b) 50 ms. c) 30 ms - STD solution at 0.5 ng/ml (30 µl - loading 6 sec.)

A typical dwell time of 100 ms was used in the regular LC-MS/MS study, but these settings were not adequate for UHPLC-MS/MS which typically generates peak widths (PW_{base}) of less than 6 seconds. The number of data points was indeed not sufficient for defining the peak apex with precision and this lead to an overestimation of the peak height (Fig. 3a). Therefore, shorter dwell times have to be used in UHPLC-MS/MS and a value of 75 ms was found adequate for the buprenorphine transition (Fig. 3b). Indeed, the chromatographic peak is well characterized without generating too many data points which creates a certain amount of noise in the chromatogram (Fig. 3c - dwell time of 50 ms).

4 Validation of a buprenorphine assay in plasma by UHPLC-MS/MS

The buprenorphine assay was validated from 0.05 to 100 ng/ml (n=5) in plasma with column-switching UHPLC-MS/MS.

Expected Concentration (ng/ml)	Sample Name	Mean (ng/ml)	Standard Deviation	%CV	Accuracy (%)
0.05	CO2	0.05	0.00	3.60	97.4
0.10	CO3	0.10	0.01	5.79	104
0.25	CO4	0.25	0.02	8.85	102
0.50	CO5	0.51	0.03	6.97	101
1.00	CO6	0.99	0.04	4.19	99.2
2.50	CO7	2.48	0.12	4.71	99.1
5.00	CO8	5.03	0.46	9.22	103
25.0	CO9	24.4	1.27	5.23	97.4
50.0	CO10	46.5	1.45	3.10	93.7
100	CO11	100	2.98	2.91	102

Expected Concentration (ng/ml)	Sample Name	Mean (ng/ml)	Standard Deviation	%CV	Accuracy (%)
0.05	QC2	0.05	0.00	4.78	109
0.10	QC21	0.08	0.01	8.86	102
0.10	QC3	0.10	0.01	6.59	102
0.25	QC4	0.25	0.02	8.17	98.2
0.50	QC5	0.49	0.03	6.19	98.5
1.00	QC6	1.05	0.04	4.26	104
2.50	QC7	2.49	0.09	3.52	99.5
5.00	QC22	5.22	0.06	1.21	104
10.0	QC8	10.4	0.40	3.86	104
25.0	QC9	24.3	0.91	3.74	97.3
50.0	QC10	47.4	1.83	3.83	95.5
100	QC11	99.1	7.98	7.92	99.1

Naltrexone and its metabolite 6-beta naltrexol were validated from 0.10 to 2.50 ng/ml (n=5) in plasma.

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