Simultaneous determination of 14 antiretroviral drugs in plasma by micro-LC-MS/MS for therapeutic drug monitoring

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Introduction

The development of combination antiretroviral (ARV) therapy had a major impact on survival rate and quality of life of HIV-infected patients. The long-term drug regimens raise some specific issues, among other adherence, virus resistance, and associated metabolic syndrome. In order to improve the treatment, these patients need close clinical and laboratory evaluations, including therapeutic drug monitoring (TDM).

The aim of our study was to develop a novel method for simultaneous determination of 14 ARV drugs (Figure 1) currently used in our hospital (atazanavir, darunavir, lopinavir, ritonavir, abacavir, emtricitabine, lamivudine, zidovudine, raltegravir, dolutegravir, efavirenz, nevirapine, etravirine and maraviroc) in plasma samples of treated patients by micro-liquid-chromatography coupled with mass spectrometry (micro-LC-MS/MS).

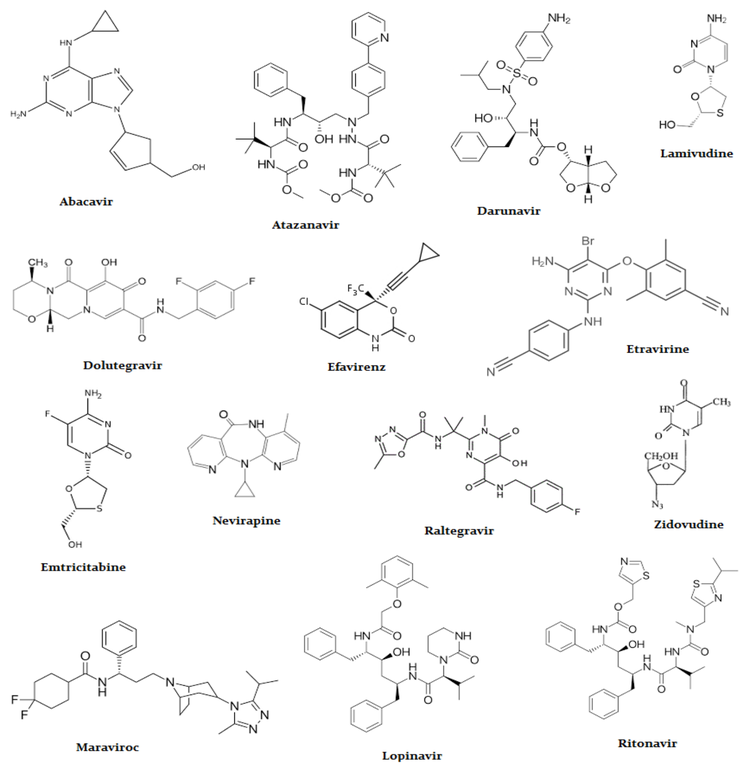

Figure 1. Structures of the investigated compounds

Analytical procedure

Sample preparation

- 50 µL of plasma/serum
- 50 µL mix of deuterated internal standards
- Protein precipitation with acetonitrile
- Vortexing, centrifugation
- 20 µL of the clear supernatant transferred in 96-well microplates and diluted with 200 µL water/methanol (9/1, v/v)
- Injection in the LC-MS/MS system

(Scheme in Figure 3.)

LC-MS/MS (CTC PAL HTS autosampler, Eksigent microLC 200, Sciex 5500 Qtrap MS)

- MS parameters optimisation for each compound
- Electrospray, positive ionisation
- Interface parameters: IS 5500V, declustering potential 100V, nebulizer and heating gas 45 psi, curtain gas 28, temperature 450°C. Acquisition parameters (scheduled MRM) are presented in Table 1.
- Chromatographic separation on Halo PFP (50 mm x 0.5 mm, 2.7 µm, 90Å)
- Mobile phase: (A) ammonium acetate 2mM in water; (B) ammonium acetate 2mM in methanol/acetonitrile (1/1, v/v), both with 0.1% formic acid
- gradient conditions; column temperature 35°C
- flow 35 µL/min, injection volume: 2 µL
- A chromatogram with all analytes is shown in Figure 2
- Calibration curves were built in plasma enriched with analyzed drugs at different concentrations, according to their pharmacokinetics. The overall range was from 1 to 15000 ng/mL.
- Quality control (QC) samples were prepared at 4 concentrations (3xLLOQ, 2 mid-range QCs and 1 high-range).

Samples

- The clinical samples were collected with informed consent from 50 HIV positive patients of the National Institute of Infectious Diseases "Prof. Dr. Matei Bals".

Drug	Q1 Mass (Da)	Q3 Mass (Da)	Retention Time (min)	CE	CXP
Abacavir	287.104	173.9	1.86	43	10
Abacavir-D4	291.1	152	1.86	43	10
Atazanavir	705.253	144.1	2.12	59	10
Atazanavir-D5	710.27	168	2.12	81	10
Darunavir	548.153	155.9	1.94	43	10
Darunavir-D9	557.209	401.2	1.94	21	18
Dolutegravir	420.039	249.1	1.92	55	14
Dolutegravir-D4	424.077	279.1	1.92	41	6
Efavirenz	316.055	53	2.15	41	8
Efavirenz-D5	321.018	246	2.15	20	14
Emtricitabine	247.987	130	1.27	21	8
Etravirine	435.045	303.9	2.12	51	18
Etravirine-D8	443.047	162.9	2.12	41	10
Lamivudine	229.956	112	1.36	19	8
Lamivudine $^{13}C^{15}N_2$	232.989	115	1.36	19	12
Lopinavir	629.279	183	2.09	40	10
Lopinavir-D8	637.254	163.1	2.09	65	10
Maraviroc	514.248	115	2.62	61	8
Maraviroc-D6	520.275	117	2.62	63	8
Nevirapine	267.086	198.1	1.7	55	12
Nevirapine-D3	270.094	229	1.7	35	14
Raltegravir	445.065	361	1.92	30	16
Raltegravir-D3	448.077	364.1	1.92	25	18
Ritonavir 2	721.169	268.1	2.06	37	10
Ritonavir-D6	727.208	146	2.06	83	10
Zidovudine	268.099	127	1.6	15	8
Zidovudine-D3	271.077	129.9	1.6	19	8

Table 1. Acquisition parameters

	Accuracy (%)				Precision (%)			
Drug	QC1 (N=17)	QC2 (N=18)	QC3 (N=18)	QC4 (N=18)	QC1 (N=17)	QC2 (N=18)	QC3 (N=18)	QC4 (N=18)
Abacavir	106.205	105.287	103.323	100.004	9.352	7.073	5.525	6.910
Atazanavir	96.707	95.186	101.925	106.361	12.477	8.032	6.205	10.572
Darunavir	112.020	106.017	101.696	100.695	9.84	9.934	8.649	9.296
Dolutegravir	110.414	108.475	104.716	103.308	9.907	6.284	6.768	7.576
Efavirenz	98.677	103.582	101.094	104.616	13.171	11.330	8.560	7.248
Emtricitabine	97.269	107.059	104.512	103.183	8.942	7.680	4.543	5.546
Etravirine	109.421	96.491	103.005	101.709	8.99	9.027	6.158	8.263
Lamivudine	97.286	110.262	105.869	101.506	9.603	2.975	4.611	5.490
Lopinavir	106.781	108.191	105.830	104.015	9.949	8.487	6.681	8.044
Maraviroc	106.768	111.847	105.448	100.442	10.615	4.941	4.576	5.876
Nevirapine	105.992	110.338	104.192	98.858	9.204	5.661	6.842	8.203
Raltegravir	102.484	114.674	109.057	97.031	11.435	4.380	6.181	12.404
Ritonavir	105.623	102.366	104.128	103.530	9.902	7.288	7.442	9.768
Zidovudine	105.621	105.602	104.598	101.681	16.916	7.532	6.870	7.452

Table 2. Validation data (accuracy and precision)

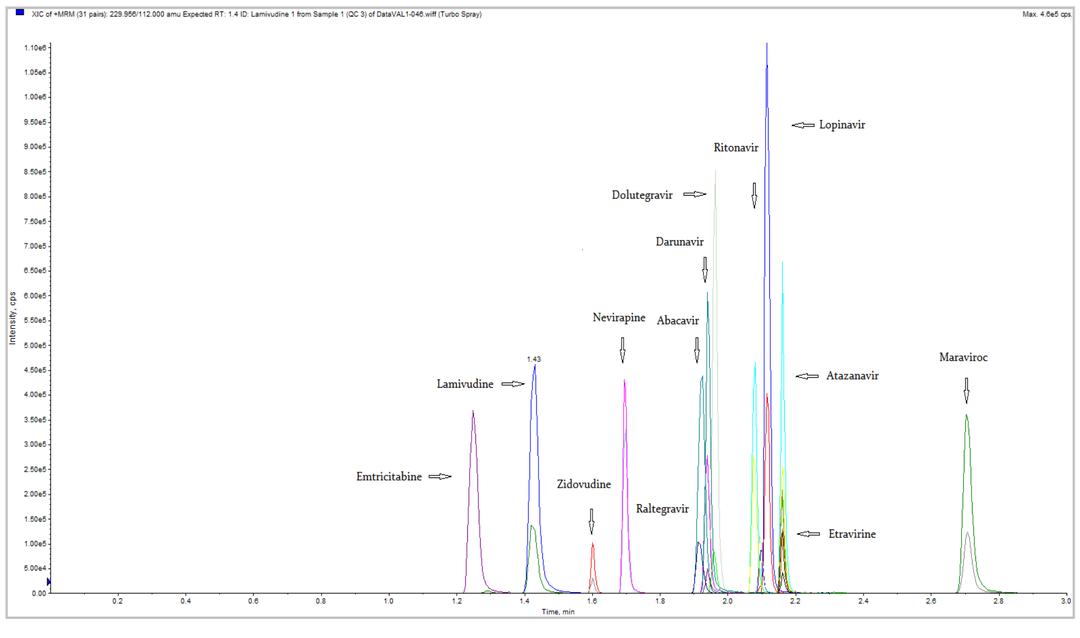

Figure 2. Chromatogram of the 14 antiretroviral drugs (QC 3)

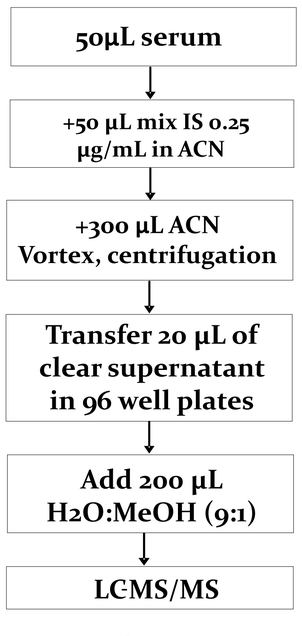

Figure 3. Schematic overview of the sample preparation protocol

Method validation

Selectivity

- Validation with added analytical standards in blank plasma
- Matrix effects assessment + Extraction recovery
- Samples from patients treated with antibiotics (e.g. vancomycin, ampicillin, linezolid, meropenem) or antiviral (sofosbuvir) were also used to test interference

Linearity

- 8 point calibration curves based on published concentrations
- Use of deuterated internal standards
- Acceptance criteria: $r^2 > 0.99$ and calibrators with acceptable accuracy and precision

Accuracy & Precision

- 4 quality control concentration levels in triplicate on 3 different days. Acceptance criteria: max ± 15% of imprecison and inaccuracy

Limit of quantification (LOQ)

- Lowest point of calibration curve. Acceptance criteria: max ± 20% of imprecison and inaccuracy
- 1 ng/mL for Abacavir, Lamividine, Zidovidine and Maraviroc

Samples stability

- Plasma 6h room temperature, 1 month -80°C, 5 freeze-thaw cycles
- Processed samples at 10°C (autosampler conditions) up to 36h

Discussion

Because of the very different polarity of the target analytes, the chromatographic separation was challenging, and after testing several stationary phases, a PFP (50x0.5 mm, 2.7 µm, 90A) column was selected. The «problematic» analytes were lamivudine (bad peak shape on C18 and other phases), and maraviroc, which tends to prefer the C18.

Calibration curves and controls were prepared in plasma at different concentrations, according to each drug's pharmacokinetics.

Issue: some drugs with high concentrations and high signal (e.g. lopinavir, darunavir), and others with low concentration and low signal (e.g. efavirenz, zidovudine). Workflow was optimized for the latter ones, and fragments with lower intensity were used when the case.

Validation was performed according to guidelines. The method has good precision and accuracy (Table 2). Matrix effects and extraction recovery were also evaluated and results were adequate (Table 3).

Samples were stable in the tested conditions (results no shown).

Application

The plasma samples from 50 treated patients were collected before ARV administration (for $C_{through}$), but also at 2, 4 and 8 hours after administration.

ARVs concentrations showed great variability among patients with the same therapy regimen. Adherence could be estimated by examining similar TDM results for the same patient in different sampling days. Three of the patients had no plasma concentrations of ARVs in the collected samples and were considered non-adherent.

Conclusion

The new method was applied in our hospital for monitoring patients with long therapeutic experience and multi-drug resistance. TDM results, together with viral load and resistance data were used for the therapeutic decisions.

	Recovery (%)					Matrix effect (%)				
Drug	QC 1 (n = 4)	QC 2 (n = 4)	QC 3 (n = 4)	QC 4 (n = 4)	Internal standard (n=16)	QC 1 (n = 4)	QC 2 (n = 4)	QC 3 (n = 4)	QC 4 (n = 4)	Internal standard (n=16)
Abacavir	74.028	74.028	101.930	97.905	86.992	62.429	79.471	93.067	105.499	90.791
Atazanavir	58.213	56.618	88.305	93.382	78.690	106.446	125.005	114.445	108.267	113.442
Darunavir	80.020	70.890	95.223	95.604	89.715	71.549	79.638	90.089	103.599	87.384
Dolutegravir	76.639	75.860	100.583	92.380	93.980	73.829	83.180	93.329	106.340	92.094
Efavirenz	89.049	79.819	93.732	97.423	0.897	61.271	67.761	97.471	100.968	87.679
Emtricitabine	82.092	86.201	86.616	87.613	108.820	71.146	66.790	65.417	75.760	69.850
Etravirine	101.787	96.104	94.349	105.279	0.8615	137.485	141.935	174.260	178.049	179.771
Lamivudine	98.626	90.357	103.983	110.968	104.275	0.614	0.640	0.882	0.901	72.895
Lopinavir	78.762	75.972	97.321	86.562	82.624	159.709	158.028	160.567	154.591	158.484
Maraviroc	64.946	61.949	84.349	81.655	74.509	60.387	66.205	81.426	95.850	75.387
Nevirapine	78.995	70.981	98.869	92.119	88.314	72.713	80.511	94.181	101.998	90.485
Raltegravir	74.717	79.812	96.488	94.830	99.660	88.354	98.871	106.095	101.401	97.820
Ritonavir	70.993	71.197	95.736	83.600	80.043	145.717	178.114	183.423	155.656	169.260
Zidovudine	84.572	73.051	98.005	96.448	90.524	72.567	80.247	92.129	99.565	88.506

Table 3. Validation data (recovery and matrix efffects)

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