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RRLC™ an alternative method to speed up the LC-MS/MS analysis, without lost of separation efficiency.

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Introduction

In the late years the progress achieved in particle chemistry which has made possible to produce phases with particle sizes of 1.8 µm and even smaller, and the development of liquid chromatography instruments capable of working at pressures higher than 400 bars, have given birth to the new techniques know as UPLC™ (Ultra Performance Liquid Chromatography, term introduced by Waters) or RRLC™ (Rapid Resolution Liquid Chromatography, term introduced by Agilent). These techniques enhance the efficiency of liquid chromatography analysis and make possible to reduce the column length and finally, to increase the mobile phase flow in order to speed up the analysis without loosing resolution and even increasing the signal to noise ratio for the peaks. [1- 3].

Columns with dimensions of 50 mm x 2.1 and 1.7-1.8 µm particle size usually work with optimum flows of 400-600 µl/min and generate pressures below 400 bars. [4,5]. A system like an Agilent 1200 HPLC, which is very similar to conventional HPLC instruments, can operate at pressure near to 600 bars, therefore such instruments can be used to enhance the laboratory throughput at a lower cost than the UPLC™ systems.

This work describes a standard fast RRLC method enables the analysis of more than 60 compounds (including anabolic steroids, anti-oestrogenics agents, β-agonists, β-blockers and stimulants) in less than 5 minutes. The power resolution of the method allows separation of structural isomers; phenylpropanolamine/catine and ephedrine/pseudoephedrine.

Experimental

The analyses were carried out with urine samples which were prepared with methanolic solutions of reference material (Table 2). Several aliquots of the spiked urine were extracted by a solid phase extraction procedure. This procedure loads 2 ml of urine throught an OASIS HLB cartridge (30 mg/ 1ml, Waters). Then the cartridge is washed with 1 ml of bidestilated water. Analites of interest are eluted with 600 µl of methanol:acetonitrile 30:70.

All analyses were performed on an Applied Biosystems API 4000Qtrap combined with Agilent 1200 HPLC system. (The analytical conditions are shown in Table 1.)

Table 1. Analytical conditions

LC conditions						
		HPLC-MS/MS	***RRLC-MS/MS***	***Quantification***		
Column		Zorbax Eclipse Plus C18 (Agilent)				
Dimensions:		**2.1 x 100mm 3.5µm**	**2.1 x 100mm 1.8µm**	**2.1 x 100mm 1.8µm**		
Mobile Phase:		A: NH_4CH_3COOH 5mM+0.1% acetic ac.		B: Acetonitrile		
Colum Temp.		50ºC				
Flow:		**300 µl/min**	**600 µl/min**	**600 µl/min**		
Gradient:		*Time*	*Time*	*Time*	*A(%)*	*B(%)*
A(%)	*B(%)*					
98	*2*	0.00	0.00	0.00	100	0
98	2	5.00	1.25	7.0	100	0
60	40	5.50	1.50			
60	40	8.00	2.00			
10	90	8.25	2.15			
10	90	12.00	2.50			
98	2	12.25	2.75			
98	2	20.00	5.00			
MS/MS conditions						
Ionization:		ESI positive		ESI positive		
Ion Spray Voltage		5500 V.		5500 V.		
Source Temp.		500ºC.		500ºC.		
Dwell Time & Scan Rate		10 msec // 0.55 scan/sec		--		
DP and CE		Specific for compound		Specific for compound		

Results and discussion

Significantly shorter analysis times were achieved with a simple change of the columns dimensions (length from 100 mm to 50 mm) and the flow rate of mobile phase (from 300 µl/min to 600 µl/min). With these modifications the total analysis time was decreased by 75% (from 20 minutes to 5 minutes). (See figure 1).

Although the systems pressures generated are elevated (350-370 bars), never exceeds the 600 bars, security threshold value for the Agilent 1200 HPLC system.

The structural isomers can be resolve in less than 3 minutes when a 1.8 µm particle size column is used. In these conditions, the chromatografic resolution is below 1.5. However, for screening purpose, the different isomers can be identified. For quantification a slight change in chromatographic conditions enables to resolve both pairs with higher resolution (figure 2 and table 1).

In theory, the use of RRLC technology should increase the signal to noise ratio. However, initially such effect was not observed (table 2). The reason was the slow scan rate programmed (0.55 scan/sec). As consequence, a poor number of scans per peak were measured.

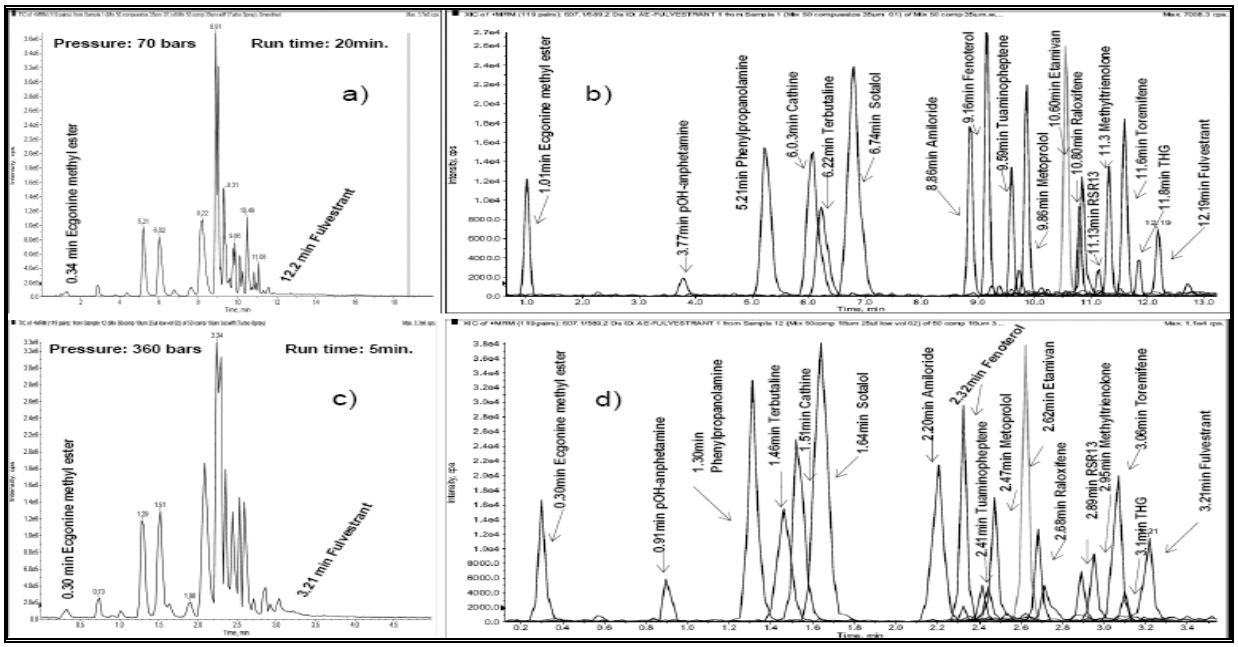

Figure 1. Comparison between conventional HPLC-MS/MS conditions and RRLC-MS/MS conditions. a and c)TIC of a mix containing more than 50 compounds analyzed by conventional HPLC (a) or by RRLC (c); b and d) extracted ion chromatogram of some of the compounds of the mix analyzed by conventional HPLC (b) or by RRLC (d).

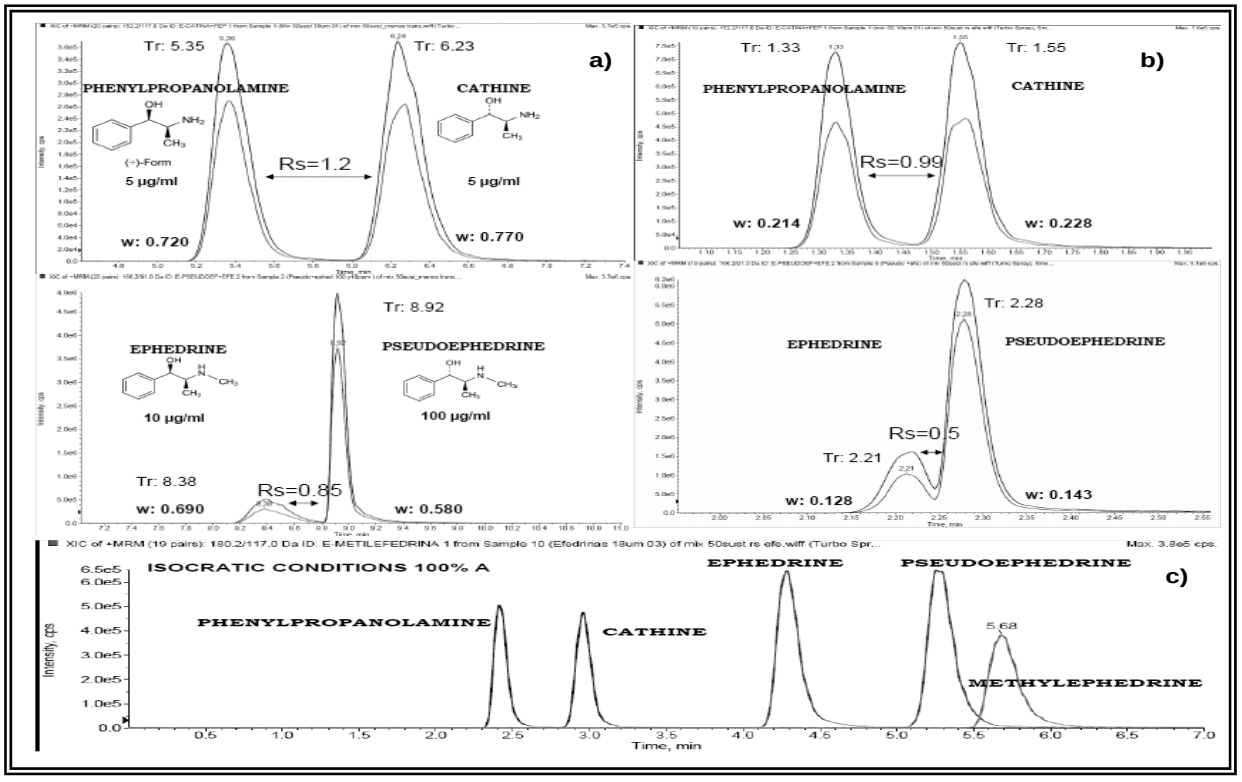

Figure 2. Resolution of isomeric pairs of phenylpropanolamine/ cathine and ephedrine/ pseudoephedrine in conventional HPLC conditions (a); in RRLC condictions (b) and in RRLC conditions slightly modified for quantitation purposes (c).

Table 2. Summary of screening data of selected compounds of the study.

Compound	*CC (ng/ml)*	*Retention Time*		*S/N*		*Peak width*		*Efficienci\y (N)*	
		HPLC	*RRLC*	*HPLC*	*RRLC*	*HPLC*	*RRLC*	*HPLC*	*RRLC*
Ecgonine Methyl ester	500	1.01	0.300	123	189	0.298	0.149	544	513
pOH anphetamine	50	3.77	0.907	142	164	0.477	0.149	3370	1316
Phenylpropanolamine	5000	5.21	1.30	4420	4440	0.745	0.209	5034	2011
Cathine	5000	6.03	1.51	3230	4220	0.864	0.268	3153	2503
Terbutaline	20	6.22	1.46	232	262	0.566	0.298	4869	2724
Sotalol	100	6.79	1.64	823	781	0.775	0.209	6030	3181
Amiloride	60	8.86	2.20	2150	927	0.328	0.238	38776	11234
Fenoterol	100	9.16	2.32	4090	2250	0.268	0.089	51926	33191
RSR13 (Efaproxiral)	2	11.1	2.89	89	160	0.268	0.209	76610	7225
Methylttrienolone	10	11.3	2.95	162	80	0.298	0.298	81762	12169
Fulvestrant	10	12.2	3.21	43	54	0.238	0.089	77864	40667

Conclusion

A fast RRLC method for the simultaneous analysis of more than 60 compounds included in the 2009 Prohibited List has been developed.

The increase in the mobile phase flow rate together with a short increase in the column compartment temperature leads to the implementation of ultrafast RRLC methods that can run multicompound analyses in less than 1 min. [3]. However, the development of these ultrafast methods requires: 1) a split of the mobile phase flow and 2) tools that allows increasing the scan rate (powerful instruments, or powerful software like MRM Schedule) in order to avoid the loss of reproducibility of the chromatographic signal reconstruction.

References

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