Fast Simultaneous Determination of the Anti-malarial Drugs, Pyrimethamine and Sulphadoxine, in Limited Volume Human Plasma Samples by LC-MS/MS

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1. Overview and introduction

Sulphadoxine, a long-acting benzene sulfonamide, and pyrimethamine, a dihydrofolate reductase inhibitor, are used as a synergistic combination in anti-malarial therapy and have nowadays replaced chloroquine as the first line anti-malarial drug, especially in Eastern Africa. In order to study the in vivo bio-availability of pharmaceutical formulations present on the African market, e.g. Fansidar® (Roche) and, as such investigate the influence of shelf time under tropical conditions on the in vivo release of both drugs, we developed and validated a fast analytical method for simultaneous quantitative determination of both drugs in limited volume human plasma samples by using LC-MS/MS. The focus was put on rudimentary, hence rapid sample clean-up and fast chromatography relying on the selectivity offered by using tandem MS. One of the main intricacies proved the quantitation of the largely different concentratrions of both compounds in one single analysis.

2. Experimental

Materials:

All solvents (water, acetonitrile (AcCN)) were HPLC-grade. Other chemicals were standard analytical grade. Pyrimethamine (PYR) and sulphamerazine (internal standard, IS) were both obtained from Sigma-Aldrich (Bornem, Belgium), while sulphadoxine (SUL) was purchased from Indis (Aartselaar, Belgium).

Ultra fast sample pre-treatment:

- Protein precipitation: 100 µL of acidified 0.1 N $ZnSO_4$-solution (pH 2.1 with formic acid) and 100 µL AcCN (containing the IS) added to only 250 µL of crude plasma
- Removal of interfering lipids: addition of 300 µL of $CHCl_3$
- Thorough mixing (15 s) and centrifugation (10 min at 4000 rpm) yielded a clear and protein-poor supernatant (10 µL injected).

LC-MS/MS conditions and apparatus:

- Column: XTerra MS C_{18} column (3.5 µm particle size, 50*1 mm) (Waters, Milford, MA, USA)
- Gradient: The mobile phase was used at a flow rate of 0.2 mL/min and consisted of a gradient of water and AcCN, both complemented with 0.5% formic acid and ammoniumformate (20 mM).
- Autosampler-Pump: Alliance 2695 Separations Module (Waters, Milford, MA, USA)
- MS: Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) used in the ESI positive ion mode, with application of MRM

Compound	Precursor ion Ion	Precursor ion *m/z*	Product ion *m/z*	Cone voltage (V)	Collision energy (eV)
PYR	$[M+H]^+$	249.1	233.1	30	30
			198.1		
			177.0		
SUL	$[M+H]^+$	311.1	245.15	70 *	15
			156.0		
			108.0		
IS	$[M+H]^+$	265.2	190.05	35	14
			172.0		
			110.0		

Table 1: Overview of the applied ESI (+) MS/MS conditions
* cone voltage detuned to extend linear dynamic range

Method validation:

- Investigated parameters: linearity, precision, accuracy, selectivity and sensitivity
- Blank plasma was fortified with both drugs: 0.1-50 µg/mL SUL
  5 to 1000 ng/mL PYR
- Quantitation: based on peak area ratios, using reconstructed mass fragmentograms (MRM-transitions underlined in Table 1)

3. Results and discussion

The applied sample clean-up approach proved extremely suitable for protein-rich biological matrices such as human plasma. Clear, protein-poor solutions are obtained without unduly dilution. Fast separation on the short Xterra column provides not only an additional clean-up but also yields separation between sulphadoxine (T_R 1.9) and pyrimethamine (T_R 2.7), as demonstrated in Figure 1. Moreover, the complete elution process only takes 6.5 min, resulting in a very fast sample turn-over and high throughput capability, which is extremely favorable in light of pharmacokinetic applications entailing a huge sample load.

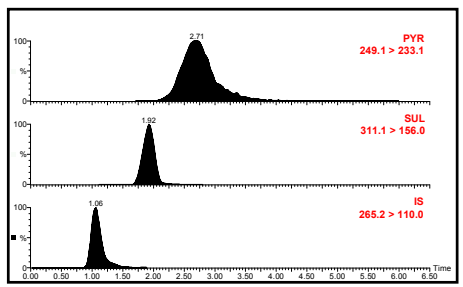

Figure 1: Extract ion chromatograms of a spiked plasma sample containing 40 ng/mL PYR and 4 µg/mL SUL.

A good linearity was obtained between 0.1 and 50 µg/mL for SUL, and between 5 and 1000 ng/mL for PYR (average R 0.9978 for SUL and 0.9984 for PYR, weighting factor 1/x, n= 5). Within-day and total reproducibility, as well as accuracy were tested at 3 different concentration levels for each analyte. The results are summarized in Table 2.

Compound	SUL			PYR		
Conc. level	µg/mL			ng/mL		
	1	10	50	10	100	1000
Total reproducibility (CV, %) (n=5)	9.8	9.7	6.5	14.9	8.5	8.2
Within-day reproducibility (CV, %) (n=7)	7.0	3.8	3.6	9.9	5.1	4.8
Accuracy (Recovery ± SD, %) (n=5)	103.7 ± 8.7	103.9 ± 6.9	100.2 ± 4.4	100.9 ± 6.2	100.7 ± 9.7	101.8 ± 4.7

Table 2: Method validation data

The coefficients of variation vary from 3.5 to 15 %, indicating a good reproducibility and accuracy over the studied concentration interval, in light of bio-analytical applications. Sensitivity of the method proved adequate with a limit of detection (LOD), using the S/N 3 criterion, of 2 ng/mL for PYR and 0.01 µg/mL for SUL. The limit of quantitation (LOQ) for both drugs was set at the lowest point of the calibration curve: 5 ng/mL and 0.1 µg/mL for PYR and SUL, respectively. Selectivity of the method was also monitored: several common sulfonamides and other drugs used in anti-malarial therapy were successfully screened for interference (plasma spiked in a concentration in large excess of the highest calibration point). Nevertheless, selectivity is guarantied not only from a chromatographic point of view, but also through selectivity offered by MRM while additionally monitored fragment ions provide a qualifier ratio. As such, adequate selectivity was proven.

4. Conclusion

In the development of the described analytical methodology the focus was put on rudimentary, hence rapid sample clean-up and fast chromatography, combined with tandem MS. The obtained validation results for biological samples clearly prove that nowadays MS-instruments achieve sufficient sensitivity in biological assays even without preconcentration step, shifting analytical challenge from sensitivity to reproducibility and carry-over issues of the whole analytical chain.

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