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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Introduction

As a result of the growing resistant falciparum malaria prevalence, the synergistic combination of sulphadoxine (SUL), a long-acting benzene sulfonamide and the dihydrofolate reductase inhibitor pyrimethamine (PYR), has replaced chloroquine as the first line anti-malarial drug in Eastern African. However, questions have arisen on the medium term stability (storage under tropical conditions), and thus bio-availability of the pharmaceutical formulations present on the African market, e.g. Fansidar® (Roche, 500 mg SUL + 25 mg PYR). After investigation of the in vitro drug release, the in vivo bio-availability needs to be investigated in an clinical trial. To that end, we developed a fast and fully validated quantitative method for simultaneous determination of both drugs in limited volume human plasma samples by using LC-MS/MS. Here, one of the main intricacies proved the quantitation of the largely different concentrations of both compounds in one single analysis.

Experimental

Materials

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

Sample pre-treatment and LC-MS/MS conditions

Ultra fast sample clean-up was achieved by protein precipitation with 100 µL of acidified 0.1 N $ZnSO_4$-solution (pH 2.1 with formic acid) and 100 µL AcCN added to only 250 µL of crude plasma, followed by vigorous shaking and centrifugation. For subsequent removal of interfering lipids 300 µL of $CHCl_3$ was added to the mixture. After thorough mixing (15 s) and centrifugation (10 min at 4000 rpm), 10 µL of the clear supernatant was injected (Waters Alliance 2695) on a Waters XTerra MS C_{18} column (3.5 µm particle size, 50*1 mm). 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). Both compounds were analysed on a triple quadrupole mass spectrometer (Micromass Quattro Ultima) in the ESI positive ion mode, by application of MRM. An overview of the applied ESI(+) MS/MS conditions for the investigated compounds is given in Table 1.

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

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

Method validation

The suitability of the developed methodology was assessed by investigating commonplace parameters in bio-analysis: linearity, precision, accuracy, selectivity and sensitivity. To that end, blank plasma was fortified with both drugs, yielding concentrations ranging from 0.1 to 50 µg/mL for SUL and from 5 to 1000 ng/mL for PYR. Quantitation was performed based on peak area ratios (SUL/IS and PYR/IS), using reconstructed mass fragmentograms after monitoring MRM-transitions as mentioned in Table 1.

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). 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.

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

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. 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.

In the development of the described analytical methodology the focus was put on the one hand on very rudimentary, hence rapid sample clean-up and fast chromatography, and on the other hand on selectivity offered by using 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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