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Short communication

Simultaneous determination of pyrimethamine, sulfadiazine and *N*-acetyl-sulfadiazine in plasma for monitoring infants in treatment of congenital toxoplasmosis

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Abstract

A method for the simultaneous determination of pyrimethamine, sulfadiazine and its metabolite *N*-acetyl-sulfadiazine in small plasma samples from neonates in treatment for congenital toxoplasmosis has been developed. In this method only 25 μl of plasma is used and a simple sample preparation based on protein precipitation and centrifugation provides highly reliable data as the recovery is about 100% and the precision is good. The analysis is performed using high performance liquid chromatography with UV and mass spectrometric (MS) detection. Pyrimethamine was found to give a linear response using MS detection in the range 0.02–5 μg/ml. Sulfadiazine and its metabolite *N*-acetyl-sulfadiazine were preferably analysed by UV at 269 nm in the concentration ranges 0.2–200 μg/ml for sulfadiazine and 0.2–50 μg/ml for *N*-acetyl-sulfadiazine.

Keywords: Congenital toxoplasmosis; Plasma concentrations; Pyrimethamine; Sulfadiazine; *N*-Acetyl-sulfadiazine; LC-MS

1. Introduction

Infection by the protozoan parasite *Toxoplasma gondii* may be transmitted during pregnancy from mother to foetus that may result in a congenital infection causing chorioretinitis, intracranial calcifications and hydrocephalus. Later in life recurrent retinochoroiditis may occur. Congenital toxoplasmosis is treated with sulfadiazine and pyrimethamine and this treatment has not changed since the end of the fifties [1]. Dose-finding studies have never been performed, and the doses used for treatment in congenital toxoplasmosis have been estimated from animal studies [2–4]. The principles for treatment of retinochoroditis with sulfadiazine and pyrimethamine was summarized by Beverly [5] in1958 and has remained almost unchanged until recent years when the use of the long-acting sulfadoxine has gained some popularity [6], however, still without any studies in animals and humans to show it is equally effective as sulfadiazine.

January 1st 1999 a national neonatal screening programme was initiated in Denmark. The screening programme is based on detection of toxoplasmosis specific IgM and/or IgA antibodies in blood obtained shortly after birth and eluted from the blood spot on the PKU filter paper (Guthrie card).

Infants with congenital toxoplasmosis are treated for 3 months with pyrimethamine 2 mg/kg/day on day 1 and then with 1 mg/kg/day on day 2 and thereafter with a maximum of 25 mg/day and sulfadiazine 50–100 mg/kg/day, but treatment efficacy has recently been questioned [7,8]. The children are furthermore treated twice a week with 7.5 mg of folinic acid for protection of the bone marrow.

One of the reasons for poor treatment outcome may be less than optimal dosing or poor compliance. In order to verify

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patient compliance analysis of plasma samples is a natural choice. However, only few and small samples may be obtained from newborns. Therefore, very little is known about plasma concentrations of these drugs in newborns.

No methods for the simultaneous determination of pyrimethamine and sulfadiazine are presented in the literature. A few methods for the determination of pyrimethamine and other sulfa drugs in plasma or whole blood using HPLC with UV detection have been published [9–16].

In the present paper, a method for the simultaneous determination of pyrimethamine, sulfadiazine and its major metabolite *N*-acetyl-sulfadiazine is described. The method involves a simple sample preparation using only 25 μl plasma and a measurement based on high-performance liquid chromatography (HPLC) coupled to a diode array detector (DAD) and a mass spectrometer (MS). Besides high sensitivity the MS technique also provides high selectivity as single ion monitoring of the three analytes and the internal standard is used. The structures of all analytes and the internal standard are given in Fig. 1. The applicability of the method is demonstrated by monitoring the plasma concentrations of the analytes in nine infants in treatment for a period of 10 weeks.

2. Materials and methods

2.1. Chemicals

Sulfadiazine and pyrimethamine were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). *N*-Acetyl-sulfadiazine was obtained from Maybridge Plc (Tintagel, UK). The HPLC column was purchased from Phenomenex (Torrance, CA, USA). All other chemicals were of analytical reagent grade.

2.2. Instrumentation

An Agilent Technologies (Waldbronn, Germany) 1100 HPLC-system equipped with a diode array detector (operated at 269 nm) and a mass spectrometer with a single quadrupole was used.

The mass spectrometer was operated in the positive electrospray mode with single ion monitoring at 249.1 mass units (pyrimethamine), 251.1 mass units (sulfadiazine), 254.1 mass units (sulfametoxazole) and 293.1 mass units (*N*-acetyl-sulfadiazine). Further settings: fragmentor: 70; V_{cap}: 2500 V; drying gas: 10 l/min; nebulizer: 25 psi g; gas temperature: 350 °C.

2.3. Chromatography

HPLC column: Phenomenex C18, AQUA, 100 mm × 4.6 mm, 3 μm. Mobile phase A: methanol + water + conc. formic acid (50:950:1, v/v/v). Mobile phase B: methanol + water + conc. formic acid (500:500:1, v/v/v). Gradient: 0–100% B 0–3 min; 100% B 3–5 min and 100–0% B 5–6 min with a total run time of 12 min. The flow rate was 0.5 ml/min and the column temperature was set to 25 °C. The UV detection was performed at 269 nm using a diode-array detector.

2.4. Sample collection and preparation

At local hospital laboratories 100–200 μl of capillary or venous blood was collected in a micro vial or in a capillary tube and centrifuged. Each sample was send by post to the analytical laboratory where it was kept at −20 °C until analysed.

Twenty-five microliters of plasma was added 25 μl of internal standard solution (60 μg/ml of sulfamethoxazole in methanol) and 20 μl of 5% perchloric acid in a 100 μl glass insert placed in an ordinary Agilent autosampler vial and supplied with a crimp cap. The mixture was carefully mixed and then centrifuged at 4000 × *g* for 15 min. Ten microliters of the supernatant was injected directly from the vial into the HPLC without disturbing the sediment (injector draw position at 12 mm).

At too high concentrations of sulfadiazine the injection volume was reduced to 2 μl.

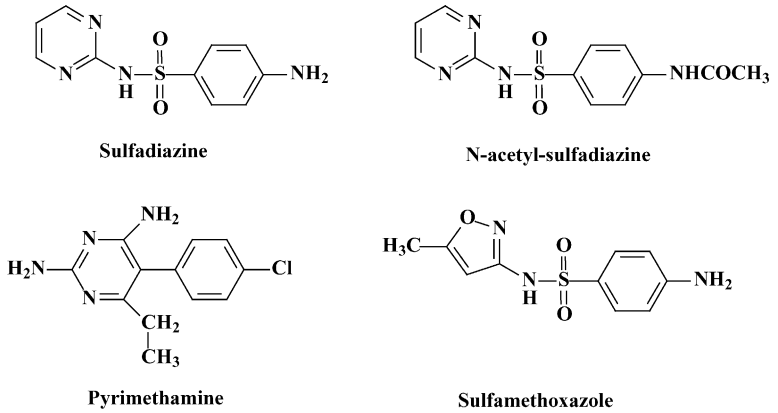

Fig. 1. Chemical structures of the analytes and the internal standard.

2.5. Calibration standards

Six calibration standards were prepared by adding the analytes to blank plasma in the concentration range 0.02–5 μg/ml for pyrimethamine, 0.2–200 μg/ml for sulfadiazine and 0.2–50 μg/ml for *N*-acetyl-sulfadiazine. These standards were analysed as described under sample preparation. The stock solutions used for the preparation of the spiked plasma samples were prepared in methanol in the concentrations 10 mg/ml pyrimethamine, 10 mg/ml *N*-acetyl-sulfadiazine and 100 mg/ml sulfadiazine.

2.6. Ethical approval

This study was approved by the Danish ethical committee (KF 11-018102).

3. Results and discussion

3.1. Chromatography

In order to achieve a good compatibility with the mass spectrometer a mobile phase consisting of organic solvent, water and formic acid was chosen from the beginning. Furthermore, sulfamethoxazole was chosen as the internal standard as this sulfa drug is not normally used in the treatment of congenital toxoplasmosis.

The sulfa drugs are easily separated in the reversed-phase system using 20% of methanol in the mobile phase, but pyrimethamine eluted with a rather long retention time. Furthermore, pyrimethamine is the analyte present in the lowest concentration and thus it was decided to use gradient elution in order to improve its detectability and shorten the analysis time. A separation of the analytes in the final separation system is shown in Fig. 2.

3.2. Detection

All the analytes may be detected and analysed by mass spectrometry. The single quadrupole used allows for single ion monitoring (SIM) detection and thus a more selective detection. However, the plasma concentrations of sulfadiazine and its metabolite *N*-acetyl-sulfadiazine in the infants are high enough to use detection by ultraviolet absorbance at 269 nm using DAD. As the calibration curves obtained using UV detection is linear this detection mode is preferred for sulfadiazine and its metabolite.

Table 2
Validation data for the three analytes obtained at three concentration levels: For pyrimethamine MS detection was used and for sulfadiazine and *N*-acetyl-sulfadiazine UV detection was used

Analyte	Added (ng/ml)	Found	R.S.D. (%) ($n = 6$)	Bias (%)
Pyrimethamine	20	19.4	6.4	−3.0
	100	97.8	3.3	−2.2
	2000	1854	2.3	−7.3
Sulfadiazine	1000	1173	1.5	17.3
	2000	1846	4.6	−7.7
	100000	91421	1.9	−8.6
N-Acetyl-sulfadiazine	200	191	10.4	−4.5
	1000	943	3.3	−5.7
	20000	16380	3.4	−18.1

3.3. Method validation

The method developed was validated with respect to linearity, recovery and precision. The linearity was tested using calibration standards at six different concentration levels. The calibration curves obtained are given in Table 1. The curves obtained are linear except for the two convex curves of sulfadiazine and *N*-acetyl-sulfadiazine obtained when using MS-detection. In all cases a good correlation between peak area of the analyte and the amount injected is obtained.

The recovery was tested at three concentration levels for each analyte and was found to be above 90% for all three analytes at all concentration levels. The investigation was performed by comparing spiked plasma samples analysed according to the method developed with solutions of the analytes in known concentrations in water and analysed directly without the sample preparation step by the HPLC method. The high recovery is due to the simple sample treatment used.

When analysing plasma samples from infants, calibration standards are prepared in blank plasma.

The precision was tested at three concentration levels and by preparing six separate samples at each concentration. In Table 2 the results are shown. The precision is good, but a bias especially for the lowest concentration of sulfadiazine is observed when using UV detection. This is due to a small blank value, which implies that the limit of quantitation for sulfa-

Table 1
Calibration curves obtained referring to the formula: $y = ax^2 + bx$, where x is the amount of the analyte and y is the area of the analyte peak in chromatograms detected either by MS or UV-DAD

Analyte	Detection	*a*	*b*	Coefficient of correlation (*r*)
Pyrimethamine	MS	0.1288	4.8146	0.9999
Sulfadiazine	UV-DAD	0.0092	1.0017	0.9999
Sulfadiazine	MS	−0.0268	0.7085	0.9981
N-Acetyl-sulfadiazine	UV-DAD	0.0315	0.9678	1.0000
N-Acetyl-sulfadiazine	MS	−0.1399	0.8241	0.9984

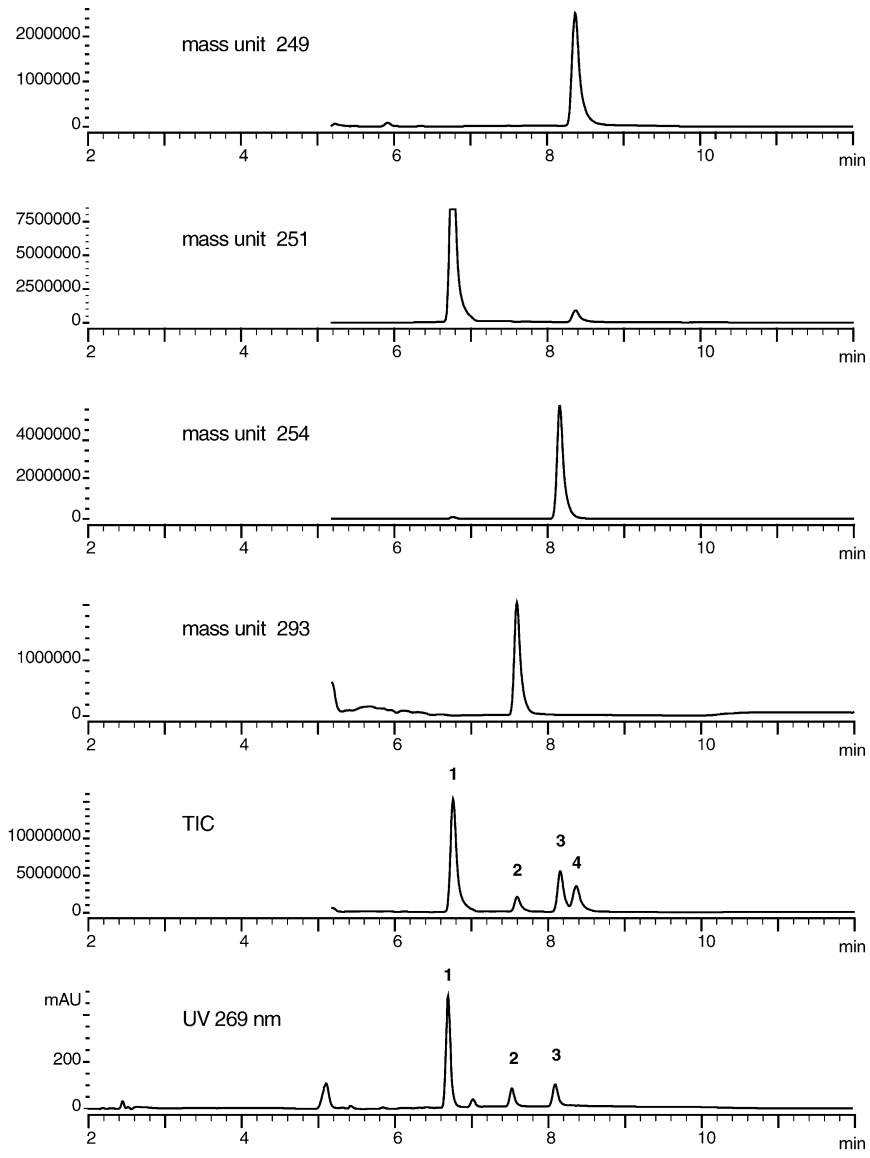

Fig. 2. Chromatogram of a typical plasma sample from an infant in treatment of congenital toxoplasmosis with pyrimethamine and sulfadiazine. The four upper traces are single ion monitoring af pyrimethamine (249), sulfadiazine (251), sulfamethoxazole (254) the internal standard and *N*-acetyl-sulfadiazine (293). The fifth trace is the total ion current (TIC) chromatogram and at the bottom the UV trace at 269 nm is given. Chromatographic conditions are given under experimental. Peak identification: 1, sulfadiazine; 2, *N*-acetyl-sulfadiazine; 3, sulfamethoxazole and 4, pyrimethamine.

diazine in plasma is 2 μg/ml. The elevated bias for *N*-acetyl-sulfadiazine is due to instability of the analyte. It was shown that *N*-acetyl-sulfadiazine after sample preparation may be hydrolysed to sulfadiazine in an amount up to 10% within 24 h. Thus, analysis has to be performed within 6 h after sample preparation.

3.4. Application

Nine infants (1–5 months old) in treatment for congenital toxoplasmosis were monitored using the method developed. The children were treated with pyrimethamine 2 mg/kg/day the first day and then 1 mg/kg/day with a maximum of 25 mg/day and sulfadiazine 50–100 mg/kg/day. The children are furthermore treated twice a week with 7.5 mg of folinic acid for protection of the bone marrow. The sulfadiazine dose was divided into two doses one given in the morning and the other given in the evening while the pyrimethamine dose was given at noon. The folinic acid was dosed together with the pyrimethamine. Blood samples were collected once a week in the morning before the first dose of sulfadiazine.

The total concentration ranges of the three analytes in the nine infants were found to be 0.8–3.0 μg/ml pyrimethamine, 35–123 μg/ml sulfadiazine and 6.0–23 μg/ml *N*-acetyl-sulfadiazine.

Typical data for two infants found over a period of 10 weeks are shown in Fig. 3. The data show that in these two cases good patient compliance is observed.

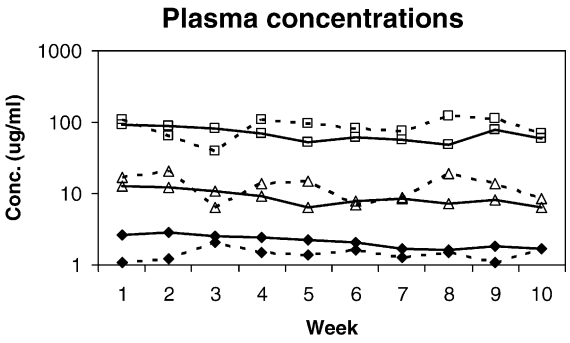

Fig. 3. Plasma concentrations obtained with the method developed in two neonates for a period of 10 weeks. Symbols: (♦), pyrimethamine; (Δ), *N*-acetyl-sulfadiazine and (□,) sulfadiazine. Full line: child number 1 and dotted line: child number 2.

The pharmacokinetics of drugs in young children and infants may differ from adults primarily due to lack of maturity of metabolising enzyme systems. Thus, children receiving long-term treatment may behave differently from adults regarding drug accumulation, metabolism and excretion of the drug and its metabolites.

Previous studies of the pharmacokinetics of sulfadiazine showed an average of the plasma concentrations between 30 and 80 μg/ml obtained after a daily intake of 400–800 mg [17,18]. These concentrations are lower than found in infants in the present study where the concentrations varied between 35 and 123 μg/ml. However, these studies [17,18] were performed in adult volunteers followed only for a few days and the data is therefore not directly comparable to data obtained for long-term treatment of children less than 6 months old.

Pyrimethamine may cause bone marrow depression and its long half-life of approximately 85 h and protein binding of 80–90% makes it important to follow its plasma concentrations. This has not been possible routinely with HPLC methods due to the large sample volume required.

The pharmacokinetics of pyrimethamine in nine children with severe congenital toxoplasmosis with hydrocephalus has been studied [19]. It was found that the plasma concentration of pyrimethamine throughout the first year of life did not vary significantly over time. Mean pyrimethamine concentration measured 4 h after drug administration was 1.3 μg/ml (S.D. 0.54 μg/ml) after dosing with 1 mg/kg body weight. Concentrations in cerebrospinal fluid were approximately 10–25% of concomitant levels in serum.

In a recent study on the pharmacokinetics of pyrimethamine and sulfadoxine in 89 children with congenital toxoplasmosis aged 1 week to 14 years a median plasma concentration of pyrimethamine of 0.1 μg/ml with ranges from 0.002 to 0.68 μg/ml [16] was found. This is considerable lower than the 0.8–3.0 μg/ml found in the present study, but pyrimethamine was administered fortnightly in the sulfadoxine study and daily in the present study, which may explain some of the difference. However, the data is difficult to compare because the age-span is 14 years in the study of Trenque et al. covering early infancy into adolescence. This makes it difficult to assess the specific problem of pharmacokinetics in infants, which is the period where most children with congenital toxoplasmosis receive continuous treatment to prevent later relapse of retinochorioiditis.

Pharmacokinetics in infants is difficult to study and especially long-term measurements in infants are important to assess compliance and to ensure that therapeutic concentrations are achieved. This is difficult with the previously published HPLC methods requiring at least 300 μl full blood.

The present method is developed to allow capillary sampling at the same time as sampling for leukocyte (WBC) and thrombocyte count performed weekly during therapy. The method requires only 25 μl whole blood in a capillary tube and can thus be obtained without additional venous sampling at the same time as a sample is obtained for the usual control of WBC and thrombocytes. Consequently, monitoring of plasma concentrations in treatment of congenital toxoplasmosis is of no further inconvenience for the infants.

4. Conclusion

The HPLC-MS method developed is accurate and precise and provides reliable data. The method opens up for detailed, longitudinal studies of children with congenital toxoplasmosis treated with sulfadiazine and pyrimethamine, which will allow treatment outcome to be linked to drug plasma levels during treatment.

It will also allow dose adjustments if too low drug plasma levels are found or reduction of doses in cases with high drug levels and leukopenia, neutropenia and thrombocytopenia.

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