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Note

Rapid method for the determination of either plasma sulphapyridine or sulphamethoxazole and their acetyl metabolites using high-performance liquid chromatography

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Sulphasalazine (SASP) has been used for many years as maintenance therapy for ulcerative colitis and more recently as a second-line agent for the management of rheumatoid arthritis (RA). SASP undergoes azo-reduction in the colon, liberating sulphapyridine (SP) and 5-aminosalicylic acid (5ASA). SP has recently been shown to be the active moiety in RA [1, 2].

A number of assays are available for the determination of plasma concentrations of SP and its metabolites using high-performance liquid chromatography (HPLC) [3–5]. The first two of these methods were found to lack both sensitivity and selectivity and have a long elution time. The third method, although highly sensitive, uses complex, expensive HPLC equipment.

The HPLC assay described here utilises standard HPLC equipment, has good sensitivity and selectivity and each analytical run is complete within 7 min allowing good sample throughput. Sulphamethoxazole (SM) is used as an internal standard. With slight adjustments to the mobile phase, this assay can be used to determine plasma concentrations of SM and acetylsulphamethoxazole (AcSM) with SP as the internal standard.

EXPERIMENTAL

Materials

Methanol, monosodium phosphate, disodium phosphate, dichloromethane and sodium acetate (all AnalaR grade) were obtained from BDH (Poole, U.K.) and acetic acid (AR) was obtained from Fisons (Loughborough, U.K.). SP, AcSP

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and 5-hydroxysulphapyridine (OHSP) were donated by Pharmacia. SM and AcSM were provided by Roche Products. β-Glucuronidase was purchased from Sigma (Poole, U.K.).

Standard solutions

Stock solutions of SP, AcSP, SM and AcSM (1 mg/ml) were prepared in methanol and stored in the dark at 4°C. Working solutions of SP, AcSP, SM and AcSM (50 μg/ml) in methanol were prepared daily.

Calibration procedure

Blank plasma samples (0.5 ml) were spiked with SP and AcSP in the concentration range 0.5–20 μg/ml suitable for single-dose pharmacokinetic studies or 2.0–40 μg/ml for steady-state concentrations. Appropriate volumes of methanol were added to ensure a constant total addition volume (200 μl), 100 μl methanolic SM (50 μg/ml) were added to all calibrations and unknown samples prior to extraction.

Extraction procedure

Methanol (200 μl) was added to 0.5 ml plasma in addition to 100 μl SM for all unknown and quality-control samples. All samples were then mixed with 1 ml of 1 *M* sodium acetate buffer (pH 4.7) and extracted into 8 ml of dichloromethane by vigorous shaking for 2 min and separated by centrifugation at 1000 *g* for 10 min at 10°C. The upper, aqueous layer was discarded and the organic layer decanted into a clean tube, evaporated to dryness under oxygen-free nitrogen at 50°C and the residue taken up into 250 μl mobile phase [methanol–0.05 *M* phosphate buffer, pH 7.4 (25:75)]. A 20-μl volume was injected onto the HPLC column.

Chromatography

The HPLC system consisted of a Constametric III dual-piston pump, a Spectromonitor III dual-cell UV detector operated at 270 nm (Laboratory Data Control), a Hewlett-Packard 3390A integrator and a Rheodyne injection system with a 20-μl loop. A Spherisorb ODS analytical column (250×3.2 mm, 10 μm particle size) (Jones' Chromatography) and a Whatman CO:Pell ODS guard column (75×4.6 mm I.D.) were used. The system was operated at room temperature with a flow-rate of 1.20 ml/min and a back pressure of 20.7 MPa.

Application of the assay

The assay has been used to analyse plasma samples from 45 RA patients receiving long-term therapy with SASP (2 g per day) or SP (1.25 g per day) and for single-dose pharmacokinetic studies following 2 g SASP (orally) in eight RA patients.

RESULTS

Extraction of plasma blanks indicated there was no interference from endogenous compounds. All peaks were well resolved as illustrated in Fig. 1. Calibration

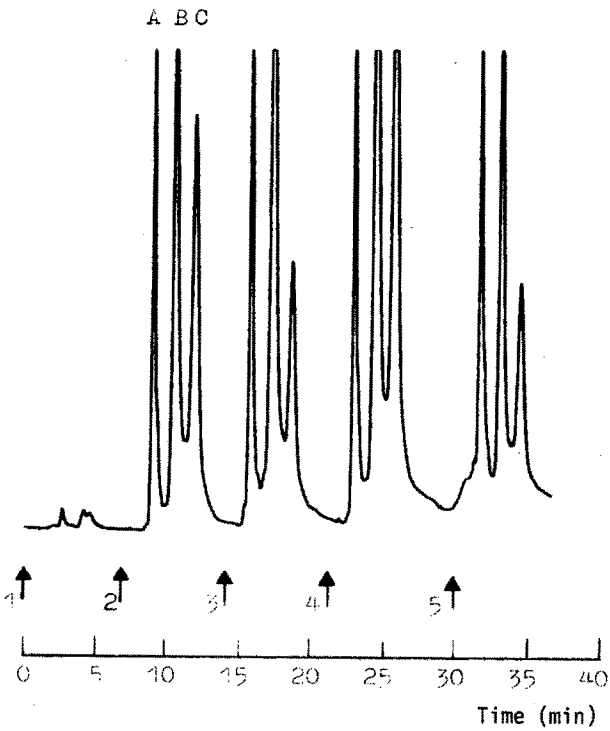

Fig. 1. SP assay. Chromatograms of blank plasma, patient plasma and standard plasma samples. (1) Blank plasma extract; (2) standard sample containing 12 μg/ml SP and 12 μg/ml AcSP; (3) plasma from a patient treated with SP (1.25 g per day); (4) standard sample containing 20 μg/ml SP and 20 μg/ml AcSP; (5) plasma from a patient treated with SASP (2 g per day). Peaks: A = SM (2.5 min); B = SP (4.2 min); C = AcSP (5.5 min).

curves of peak-height ratio versus plasma SP and AcSP concentration were linear and reproducible. The mean correlation coefficients for SP and AcSP calibrations obtained from fifteen analytical runs were 0.9981 and 0.9966, respectively. The inter-batch precision and accuracy of the assay were determined from a mean of the back-calculated standards and are shown in Table I. Quality-control samples gave concentrations of SP and AcSP within ±15% of a pre-determined mean value. Recovery was determined by comparing the extraction from plasma spiked with SP and AcSP standards (10 μg/ml) with the equivalent concentration of

TABLE I

PRECISION, ACCURACY AND RECOVERY OF SP, AcSP, SM AND AcSM

Compound	Precision (%)	Accuracy (%)	Recovery (%)	r (mean)
SP	5.1	102	83	0.9981
AcSP	9.4	101	84	0.9966
SM	3.9	102	84	0.9987
AcSM	5.8	101	82	0.9981

standard solution in methanol, added to an extract of blank plasma, blown-dry and re-dissolved directly into mobile phase. In both cases, internal marker was added prior to extraction. The results are shown in Table I.

Steady-state plasma concentrations of SP and AcSP of up to 40 μg/ml were detected following 2 g per day SASP or the molar equivalent of SP (1.25 g per day) and were dependent on acetylator status. Slow acetylators had high concentrations of SP and low concentrations of AcSP, the reverse was true for fast acetylators.

Peak concentrations of up to 15 μg/ml SP or AcSP were found 15–16 h after the administration of a single 2-g dose of SASP orally.

The addition of SASP to blank plasma and standard samples indicated that it was not detected on the chromatogram. This drug has maximum UV absorbance at 360 nm and has a poor recovery when extracted into dichloromethane. Non-steroidal anti-inflammatory agents were found not to interfere with the assay. Calibration experiments showed that OHSP eluted between SM and SP with a retention time of 3.1 min. However, this species was not detected in plasma samples from patients. Incubation of samples with β-glucuronidase (50 U at 37°C, overnight) failed to increase concentrations of SP, OHSP and AcSP.

Determination of plasma SM and AcSM concentrations

The same extraction procedure as described above was employed using 100 μl plasma and 100 μl SP (50 μg/ml) as internal standard. A calibration range of 10–100 μg/ml SM and 2.5–25 μg/ml for AcSM was used. The mobile phase was methanol–0.05 *M* phosphate buffer, pH 7.4 (20:80).

Resolution of the peaks was good with no interfering endogenous compounds appearing on the chromatogram (Fig. 2). Reproducible linear calibrations of peak-height ratio versus plasma SM and AcSM concentrations were obtained. The mean correlation coefficients, recovery and inter-batch precision and accuracy are detailed in Table I. Quality-control samples gave concentrations of SM and AcSM within ±15% of a pre-determined mean value.

Patients receiving steady-state therapy with 1.4 g per day co-trimoxazole SM–trimethoprim (5:1) were found to have plasma concentrations of up to 100 μg/ml SM and up to 25 μg/ml AcSM. Trimethoprim was found not to interfere with the assay.

DISCUSSION

This paper primarily describes the HPLC assay of plasma SP and AcSP concentrations; however, with minor adjustments to the mobile phase, the method becomes suitable for plasma SM and AcSM determination. Although the latter assay may not be as sensitive as previously published methods which determine trimethoprim, SM and AcSM simultaneously [6, 7], it provides a rapid, cost-effective method for the determination of plasma concentrations of more than one sulphonamide and their acetyl metabolites.

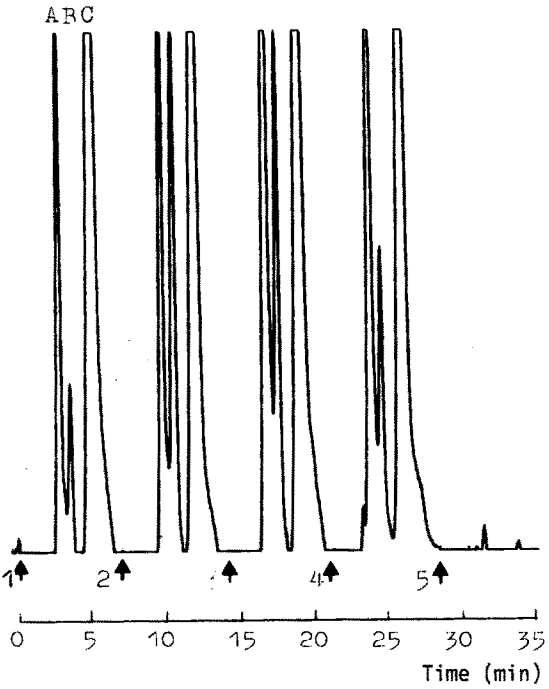

Fig. 2. SM assay. Chromatograms of blank plasma, patient plasma and standard plasma samples. (1) Standard sample containing 20 μg/ml SM and 5 μg/ml AcSM; (2 and 4) plasma from two patients treated with 1.4 g per day co-trimoxazole; (3) standard sample containing 60 μg/ml SM and 15 μg/ml AcSM; (5) blank plasma extract. Peaks: A = SM (2.7 min); B = AcSM (3.7 min); C = SP (5.2 min).

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