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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF PYRIMETHAMINE, SULFADOXINE AND ITS N^4-ACETYL METABOLITE IN SERUM AND URINE AFTER INGESTION OF SULDOX

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SUMMARY

A sensitive and selective reversed-phase high-performance liquid chromatographic assay has been developed to determine the concentration of pyrimethamine, sulfadoxine and N^4-acetylsulfadoxine in serum and urine after oral administration of the antimalarial remedy Suldox®. Hitherto the literature describes no method being able to quantitate all three compounds in these fluids.

The compounds are extracted successively from the same sample and subjected to liquid chromatography followed by ultraviolet detection (280 nm). Calibration curves were linear ($\overline{r^2}$ = 0.999; S.E.M. less than 3%; n = 10) in the range 0—300 μg/ml (sulfadoxine) and 0—1000 ng/ml (N^4-acetylsulfadoxine and pyrimethamine). The limits of quantitation for the latter compounds were as low as about 5 ng/ml and 1 ng/ml, respectively. At therapeutic serum concentrations of 30 μg/ml (sulfadoxine), 350 ng/ml (N^4-acetylsulfadoxine) and 120 ng/ml (pyrimethamine) an interassay reproducibility below 8% (relative standard deviation) was found for all three compounds.

The assay was evaluated in a pilot study and proved convenient for pharmacokinetic studies in man following oral co-administration of pyrimethamine and sulfadoxine.

INTRODUCTION

Sulfadoxine (SDP) and pyrimethamine (DCE) are both long-acting drugs inhibiting sequential steps in the biosynthesis and utilization of folic acid [1]; used together they act synergistically [1, 2]. Given as a single drug SDP has been used in the treatment of various infections [3—6]. DCE monotherapy has been used in the treatment of meningeal leukaemia [7, 8], malaria due to *Plasmodium falciparum* [9, 10] and against coccidiosis [11, 12]. The fact that the concurrent use of DCE and SDP causes a double blockade in the folate pathway has been beneficial for the treatment and prophylaxis of protozoal

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diseases such as malaria tropica when caused by chloroquine- and/or pyrimethamine-resistant strains of *Plasmodium falciparum* [13—15].

Spectrophotometric methods were used for the analysis of both DCE [16, 17] and SDP [18, 19] until the late sixties. The first gas chromatographic method for DCE [20] dealt with its concentration in chicken tissue. Later, gas—liquid chromatographic (GLC) methods for DCE analysis in plasma were published [21–23]. The minimum concentration measurable in all cases was about 5 ng/ml. Disadvantageous to these techniques were a cumbersome procedure, a long time of chromatography [21] and the occurrence of ghost plasma peaks [22]. Two liquid chromatographic methods for DCE in plasma have been published [24, 25] and both signified simplifications of the procedure. A GLC method feasible for SDP in urine and whole blood was published in 1981 [23]; however, the chromatography involved a time-consuming temperature programming of the column oven. Some high-performance liquid chromatographic (HPLC) methods for sulphonamides in biological fluids have appeared [26—28], but to the authors knowledge none on SDP.

This publication describes a reversed-phase HPLC method to determine therapeutic concentrations of DCE, SDP and its N^4-acetyl metabolite (MAS) in plasma and urine after intake of Suldox® (Dumex). This antimalarial product contains DCE and SDP in the ratio of 1:20.

EXPERIMENTAL

Chemicals and reagents

SDP, MAS, DCE and the internal standards monobutyrylsulfadoxine (MBS) and *p*-aminopropionphenone (PAP) were supplied by the Synthesis Laboratory, Dumex. Acetonitrile was of HPLC grade and all other chemicals were of analytical grade.

Standard solution I: 10 mg of DCE and 10 mg of MAS were dissolved in 100 ml of methanol.

Standard solution II: 20 mg of SDP and 1 ml of standard solution I were added to 100 ml of methanol giving the concentrations 200 ng/μl SDP, 1 ng/μl MAS, 1 ng/μl DCE.

Internal standard solution I: 10 mg of MBS were dissolved in 100 ml of methanol.

Internal standard solution II: 10 mg PAP were dissolved in 100 ml of methanol.

Borate buffer (0.0125 *M*) resulted from the dissolution of 4.8 g of $Na_2B_4O_7 \cdot 10H_2O$ in 1 l of glass-distilled water, addition of 1.5 g of sodium hydroxide and final pH adjustment with 2 *M* sodium hydroxide to pH 10.0.

TBA reagent (0.25 *M*): 8.5 g of tetrabutylammonium hydrogen sulphate were dissolved in 100 ml of glass-distilled water and the pH was adjusted to 10.0 with 2 *M* sodium hydroxide

Sodium hydroxide (2 *M*): 8 g of sodium hydroxide were dissolved in 1 l of glass-distilled water.

Sodium phosphate buffer (0.1 *M*): 18.0 g of $Na_2HPO_4 \cdot 2H_2O$ were dissolved in 1 l of glass-distilled water and concentrated orthophosphoric acid was added to pH 4.0 or 5.4.

For hydrolysis of urine samples β-glucuronidase (EC 3.2.1.31) from Sigma was used.

Mobile phase for chromatography of SDP and DCE in serum and urine (mobile phase A): a mixture of equal volumes of acetonitrile and sodium phosphate buffer (0.1 *M*, pH 4.0)

Mobile phase for the chromatography of MAS in serum (mobile phase B): acetonitrile and sodium phosphate buffer (0.1 *M*, pH 5.4) were mixed (30:70, v/v).

Mobile phase for the chromatography of MAS in urine (mobile phase C): acetonitrile and sodium phosphate buffer (0.1 *M*, pH 5.4) were mixed (20:80, v/v).

The mobile phases were degassed before use by ultrasonication.

Chromatographic instrumentation

Liquid chromatography was performed on a Waters Model 6000A constant-flow solvent delivery system, a Waters U6K injection unit and a Waters Model 440 ultraviolet (UV) detector equipped with a 280-nm filter. The following 5 μm column packing materials were used: spherisorb S5 Phenyl, Spherisorb S5 Octyl and Spherisorb S5 ODS (Phase Separations, U.K.). The stainless-steel columns (Herbert Knauer, F.R.G.), 25 cm × 4 mm I.D., were packed using a downward slurry technique employing isopropanol and methanol. Detector response was monitored with a Hewlett-Packard Model 3380 integrator. A water-bath was used to maintain column temperatures of 20°C and 30°C.

Extraction from human serum and urine

The extractions of the three compounds (Fig. 1) were performed on the same sample. When internal standards for SDP and/or DCE were used, 80 μg of MBS (i.e. 800 μl of internal standard solution I) and/or 1500 ng of PAP (i.e. 15 μl of internal standard solution II) were added to 1 ml of the intact sample.

Pyrimethamine (DCE). A 1-ml sample was added to 3 ml of borate buffer (pH 10) and mixed. Then 5 ml of diethyl ether were added and after shaking for about 10 sec and centrifugation, 4 ml of the supernatant were transferred to a conical tube and evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of mobile phase A and 40 μl were injected on to the column.

Sulfadoxine (SDP) and N^4-acetyl sulfadoxine (MAS). The aqueous phase remaining from the ether extraction of DCE was subjected to a nitrogen jet stream to evaporate residual diethyl ether. Then 1 ml of TBA reagent was admixed, thus using the tetrabutylammonium ion as an ion-pairing agent. After the addition of 5 ml of dichloromethane the tube was rotated for 3 min and centrifuged at 10°C. The aqueous supernatant was then removed using a pasteur pipette. At this point the procedure divided into separate routes for MAS and SDP. (1) A 3-ml portion of the remaining organic phase was transferred to a conical tube and evaporated with a nitrogen jet stream. The residue was dissolved in 100 μl of mobile phase B (serum) or mobile phase C (urine); 40 μl were injected on to the column for analysis of MAS. (2) From the remaining organic phase 20 μl (serum) or 200 μl (urine) were transferred to conical tubes. After evaporation with nitrogen the residue was redissolved in 200 μl of mobile phase A and 40 μl were injected on to the column for analysis of SDP.

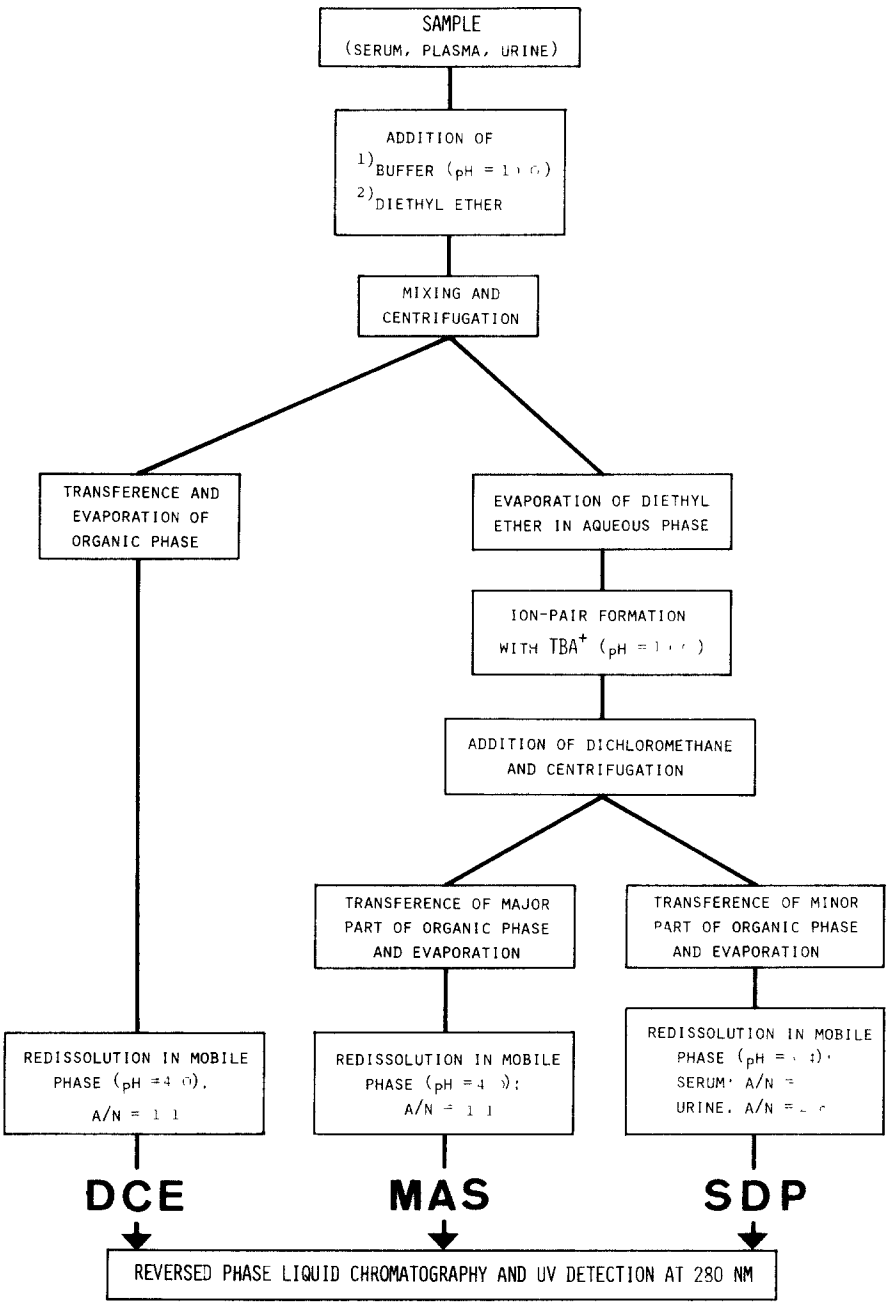

Fig 1. Flow-chart of analytical procedures to quantitate sulfadoxine (SDP), N^4-acetylsulfadoxine (MAS) and pyrimethamine (DCE) in biological fluids. TBA^+ = tetrabutylammonium ion; A/N = volume ratio of acetonitrile and sodium phosphate buffer.

Liquid chromatography

The different mobile phases used were delivered through Spherisorb S5 ODS at a flow-rate of 1 ml/min giving in all cases a pressure drop of about 100 bar per 25 cm. The temperature of the column and of the solvent was thermo-

statically maintained at 20° C, except for chromatography of MAS in urine for which the temperature was 30° C.

Calibration curves

Standard samples were prepared by evaporating 0—1500 μl of standard solution II and volumes of the internal standard solutions I and II as mentioned above. To these residues were added 1 ml of drug-free urine or serum and these samples were then subjected to the procedure described above. The calibration curves were obtained by plotting the peak area ratios for DCE/PAP and SDP/MBS against concentration; for MAS the peak area was used. The curves were calculated according to linear regression by means of the method of least squares.

Quality control

To estimate the precision and accuracy of the analyses, quality control samples were prepared and analysed along with the biological samples. The pools were prepared by addition of the analytes to human blank serum and following gentle shaking the mixture was dispensed into tubes in 1-ml aliquots and stored at −20° C.

RESULTS AND DISCUSSION

Chromatographic conditions

Reversed-phase mode was preferred because a low retention of MAS relative to SDP was desired. This was considered beneficial for the detection sensitivity in that the plasma concentration of MAS was several orders of magnitude lower than that of SDP. C_8 and C_{18} bonded phases proved to be equally useful for the chromatography and an acceptable column performance was observed for about 600 succesive injections.

The components of the mobile phase were finally settled as acetonitrile and phosphate buffer. During the development work ion-pair chromatography for SDP and MAS was also tried with tetrabutylammonium hydrogen as the ion-pairing agent, but this was unsatisfactory regarding peak shapes. For the chromatography of MAS in urine the temperature of the mobile phase was increased from 20° C to 30° C to provide the required lower retention of endogenous urinary constituents relative to MAS.

The analysis of DCE in both serum and urine was selective and no peaks suspected of being metabolite peaks appeared. However, during the chromatography of about 600 serum samples including standards a small peak cluster was sometimes observed shortly after the peak of DCE. Occasionally it partly overlapped the peak of DCE (Fig. 2); thus peak heights were used rather than areas in the calculation of concentration.

Examples of typical serum and urine tracings are shown in Figs. 2 and 3. The capacity ratios (and theoretical plate numbers) were as follows: SDP, 3.5 (6000); MBS, 5.0 (5000); MAS (serum), 5.1 (6100); MAS (urine), 8.8 (5900); DCE, 7.0 (4300); PAP, 5.4 (4500). The peak resolution of SDP and MBS could be calculated as 1.8 from the serum chromatograms (Fig. 2).

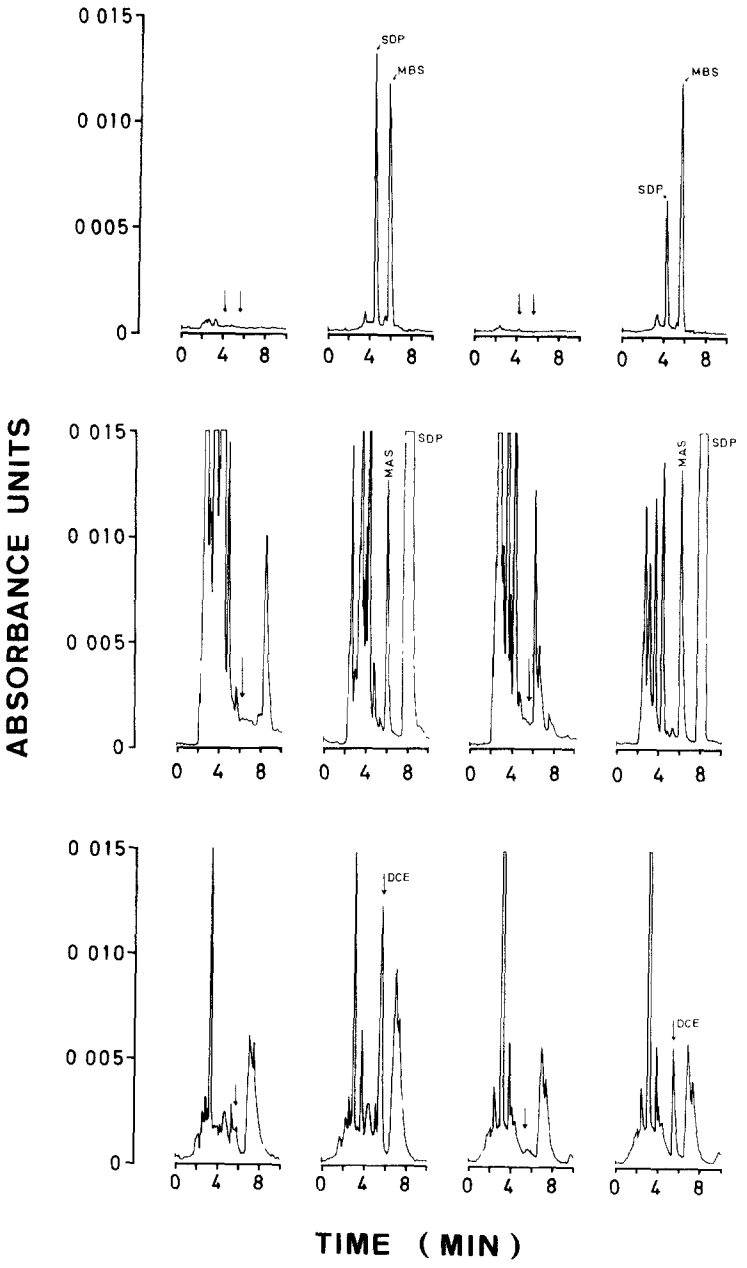

Fig. 2. Chromatograms of human serum extracts of 1-ml samples. UV detection at 280 nm. The arrows indicate retention times of the analytes. Each panel shows the following tracings (from left to right): blank serum standard, serum standard, volunteer serum prior to dosing, volunteer serum collected after a single oral dose of Suldox (25 mg DCE and 500 mg SDP). Peaks in the non-blank standard and volunteer serum tracings represent in the upper panel· 40 μg SDP and 55 μg MBS (standard), 18 μg SDP and 55 μg MBS (volunteer serum); central panel: 80 ng MAS (standard) and 85 ng MAS (volunteer serum); lower panel: 80 ng DCE (standard) and 30 ng DCE (volunteer serum).

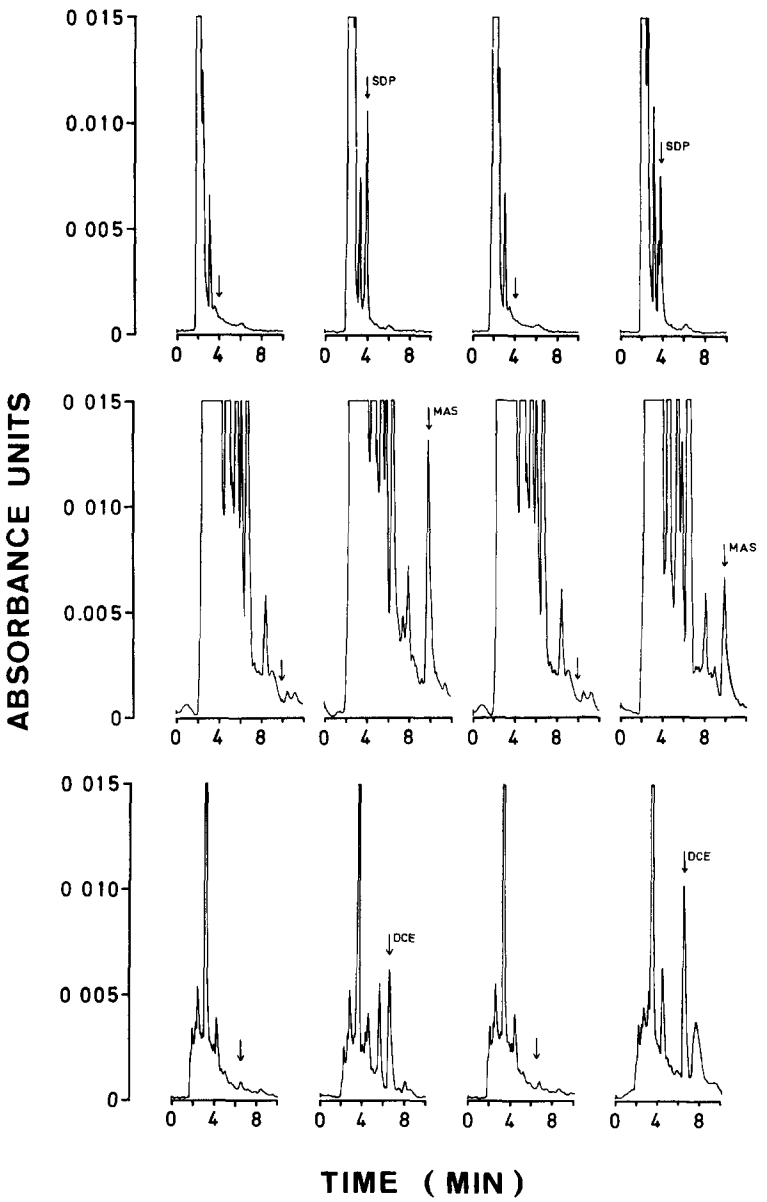

Fig. 3. Chromatograms of human urine extracts of 1-ml samples. UV detection at 280 nm. The arrows indicate retention times of the analytes. Each panel shows the following tracings (from left to right): blank urine standard, urine standard, volunteer urine collected after a single oral dose of Suldox (25 mg DCE and 500 mg SDP). Peaks in the non-blank standard and volunteer urine tracings represent in the upper panel: 40 μg and 28 μg SDP; central panel: 80 ng and 35 ng MAS; lower panel: 40 ng and 60 ng DCE.

Choice of internal standards

Internal standards for SDP and DCE were chosen as mentioned above. If a smaller amount of MBS was added it could apply for MAS instead of SDP. The internal standard for DCE (PAP) was sometimes not satisfactory because

of interfering constituents in serum and urine (Figs. 2 and 3). This interference amounted to about 5% of about 600 samples from twenty subjects. As a consequence PAP was excluded from routine work. The internal standard for SDP (MBS) was used throughout for analysis of serum. For SDP in urine, however, the applicability of MBS was verified but not used routinely.

Choice of extraction procedures

The final extraction procedure (Fig. 1) utilized a priori diethyl ether extraction of DCE from the alkalinized samples and ion-pair extraction of SDP and MAS from the remainder. It was found that addition of 85 mg of TBA dissolved in 1 ml of water was sufficient for optimum ion-pair formation as calculated per 1 ml sample and in the presence of the internal standard MBS as specified above (Experimental).

Extraction yields were assessed by injection of known amounts of the analytes on to the column and similarly for processed samples. The ratio of the slopes of these two linear correlations was used as an estimate of the extraction recovery for each analyte. It appeared that the extraction yields from urine were fully quantitative for MAS, MBS, PAP and DCE. Urine SDP was extractable to 83%. A lowering of the extraction yields resulted when serum concentrations were examined: 67% for SDP, 94% for MAS and MBS, 96% for PAP, and 90% for DCE. All extraction yield experiments were processed in quadruplicate and the S.E.M. was in all cases less than 3%.

Quantitation and reliability of the analytical results

Linearity of the calibration curves were established as described above in the concentration range 0—300 μg/ml (SDP) and 0—1000 ng/ml (MAS, DCE). For all three compounds a determination coefficient was found $\bar{r}^2 = 0.999$ ($n = 10$) with S.E.M. less than 3%. The ordinate intercepts did not deviate significantly from zero. The observed S.D. for the three analytes in the serum pools was in all instances within 8% of the mean ($n = 10$). The mean concentrations were 30 μg/ml (SDP), 350 ng/ml (MAS) and 120 ng/ml (DCE), i.e. within the therapeutic concentration range found (see below). An increase of the inter-assay reproducibility was seen when the internal standards were excluded from the calculations.

Limits of quantitation

The methods described for MAS and DCE had lower limits of quantitation of about 5 ng/ml (MAS) and 1 ng/ml (DCE). These limits resulted from optimization of the volume taken in the extraction as well as the volume injected on to the column. The reproducibilities of the assays seemed unaffected by such adjustments. A signal-to-noise ratio of about 2:1 was observed for these minimum quantifiable concentrations. Such a limit for the analysis of SDP was not relevant because the therapeutic range was several orders of magnitude above this limit.

Storage of urine and serum samples

Samples from the pilot study (see below), drug-free serum for standards and quality control pools were stored for about six months at −20° C and prior to

analysis 1—2 h at ambient temperature. This mode of storage was observed to be without significant detriment to the stability of the drugs. In addition no deterioration was noticeable for serum and urine samples undergoing two freeze—thaw cycles.

Analytical interferences

None of the following drugs interfered with the determination of SDP, MAS and DCE in serum or urine: acetylsalicylic acid, diazepam, quinine, chloroquine.

Application of the methods

Serum and urine samples from a 39-year-old female volunteer were successfully applied to demonstrate the validity of the methods. The samples were

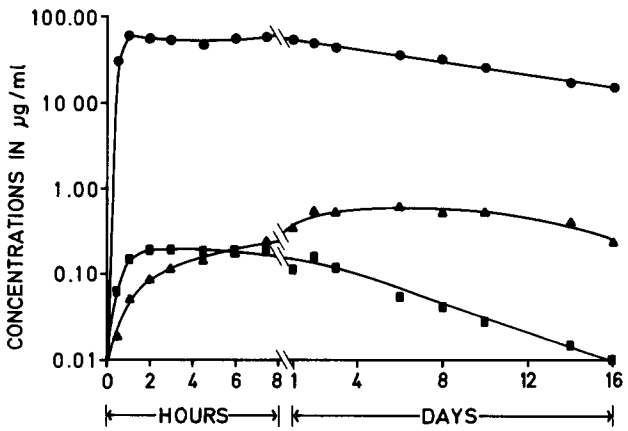

Fig. 4. Serum concentration profiles of the three analytes studied obtained following a single oral administration of one tablet of Suldox (25 mg DCE and 500 mg SDP) to a 39-year-old female volunteer. (•), SDP; (▲), MAS; (■), DCE.

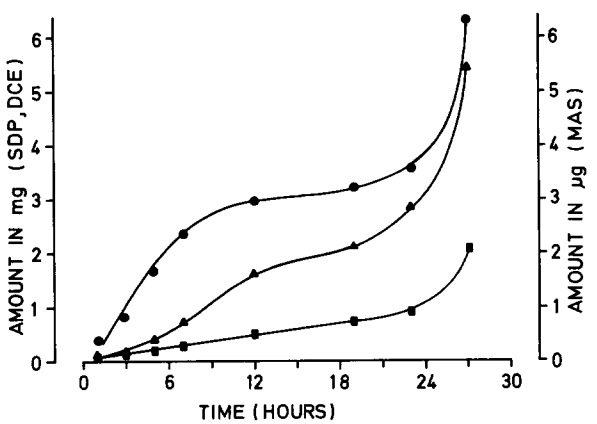

Fig. 5. Cumulative urinary excretion profiles of the three analytes studied obtained following a single oral administration of one tablet of Suldox (25 mg DCE and 500 mg SDP) to a 39-year-old female volunteer. The times of the data points are the midpoints of the collection time periods. (•), SDP; (▲), MAS; (■), DCE.

taken after oral administration, in a fasting state, of one tablet of Suldox containing 25 mg of DCE and 500 mg of SDP. The serum results for DCE, SDP and MAS (Fig. 4) show elimination half-lives from the circulation of about 100 h (DCE) and 190 h (SDP). The findings agree with the sparse pharmacokinetic data available in the literature on DCE [24, 29] and SDP [30]. Hitherto, lack of an analytical method has precluded knowledge about plasma levels of MAS.

Fig. 5 shows the accumulated urinary excretions. The data points are plotted in the midpoints of the collection time periods. It is evident that the excretion rates still increase at the time of the last data points. Within the collection period of 31 h about 1% of SDP and about 8% of DCE was recovered in the urine. The latter results compare well to a previous finding in these laboratories using a gas chromatographic method [22]. The urine samples were subjected to enzymatic hydrolysis with β-glucuronidase. Analysis of the hydrolysed samples showed the presence of at most 5% SDP and MAS glucuronides in the urine.

No comparisons to other estimations are feasible via the literature as regards the urinary excretion of SDP and MAS.

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