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Determination of sulfadoxine in human blood plasma using packed-column supercritical fluid chromatography

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Abstract

A sensitive, rapid, selective and reproducible method has been developed to measure plasma levels of sulfadoxine, 4-Amino-*N*-(5, 6-dimethoxy-4-pyrimidinyl) benzensulfonamide; in healthy, human volunteers using packed-column supercritical fluid chromatography. Omeprazole, 5-methoxy-2-[[(4-methoxy-3, 5-di-methyl-2-pyridinyl)methyl]sulfinyl]-1H-benzimidazole; was used as the internal standard (i.s.) at 15.0 μg/ml. The drug and the i.s. were extracted from plasma using dichloromethane. Separation of sulfadoxine and i.s. was done on a Nucleosil (250×4.6 mm) 10 μm, RP-C_{18} column with 7.4% (v/v) methanol-modified supercritical fluid carbon dioxide (2.5 ml/min) as the mobile phase. The column temperature was 40°C and the outlet pressure was set at 8.83 MPa. The detection was done using a UV–Vis detector set at 265 nm. The limit of quantification was 0.50 μg/ml using 1 ml plasma specimen. The mean extraction recovery of the drug from plasma was found to be 94.9%. The SFC method was directly compared to a published HPLC/UV method. With respect to speed and use of organic solvents SFC was found to be superior; while in all other aspects the results were similar to the published technique. The method has been successfully used to estimate the sulfadoxine levels in healthy human volunteers from 0 to 240 h following an oral dose of 500 mg of sulfadoxine in combination with 25 mg of pyrimethamine.

Keywords: Sulfadoxine

1. Introduction

Sulfadoxine, (Fig. 1), 4-amino-*N*-(5, 6-dimethoxy-4-pyrimidinyl) benzene sulfonamide; is a long acting sulfonamide used in synergistic combination with pyrimethamine, a dihydrofolate reductase inhibitor, to treat chloroquine resistant falciparum malaria. It is also used for prophylaxis in areas where chloroquine resistant falciparum malaria is prevalent [1]. Several methods have been reported for the estimation of sulfadoxine in biological fluids, including gas chromatography [2], as well as HPLC techniques [3–6].

Traditionally, the pharmaceutical industry has employed reversed-phase HPLC with UV-absorbance detection as the analytical method of choice. Packed column SFC is a relatively new technique in which a pressurised gas under certain conditions of temperature and pressure acts as a fluid and can be used as a mobile phase as is or by changing its polarities with a suitable modifier like methanol or acetonitrile. The

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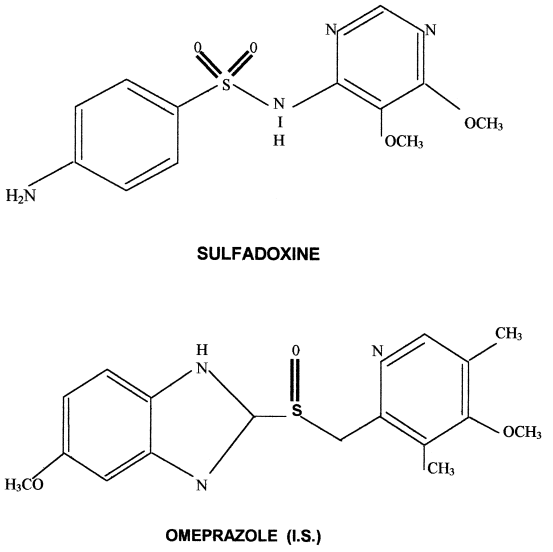

Fig. 1. Structures of the drug and the internal standard (i.s.).

present interest in packed-column supercritical fluid chromatography (PCSFC) for the estimation of drugs from biological fluids stems from a series of publications [7–11] in which the authors have claimed superior, or at least equal chromatography compared to standard liquid chromatography (LC). The low viscosities and high diffusivities of supercritical fluids enhance chromatographic efficiencies. Further, SFC generates less solvent waste and employs a cheap non-toxic gas, carbon dioxide. The present work describes a rapid, sensitive, simple, reproducible and specific PCSFC technique for the estimation of sulfadoxine in human blood plasma using omeprazole (Fig. 1) as an internal standard.

2. Experimental

2.1. Chemicals and reagents

The working standards of sulfadoxine, omeprazole and pyrimethamine were obtained from M/s Ipca Laboratories Ltd., Mumbai, India, while those of fluconazole, lansoprazole, sulfaguanidine and sulfamethoxazole were obtained from M/s Macleods Pharmaceuticals Ltd. Mumbai, India. The samples were also assayed by official methods [12] in this laboratory. Methanol and acetonitrile (HPLC grade), and dichloromethane, potassium dihydrogen orthophosphate, glacial acetic acid, perchloric acid and orthophosphoric acid (85%) (all AR grade) were purchased from E. Merck (India), Mumbai, India. Carbon dioxide was 99% pure obtained from Bombay Carbon Dioxide Co., Mumbai, India. A buffer solution was prepared by mixing 9.9 ml of 0.02 *M* KH_2PO_4 (pH 3.4) with 0.1 ml of glacial acetic acid.

2.2. Preparation of standard solutions

Individual drug solutions were prepared by weighing 100 mg of each drug and dissolving it in 100 ml of methanol to give concentrations of 1 mg/ml. Stock solutions of sulfadoxine and the i.s. were then diluted with methanol to appropriate concentrations (5.0–1000 μg/ml for sulfadoxine and 150 μg/ml for i.s.). The stock solutions of the other test drugs were also diluted to 100 μg/ml. All these solutions were stored in the refrigerator.

For control plasma, fresh frozen human plasma received from a blood bank (Green Cross, Mumbai, India), was thawed at room temperature and centrifuged at 1000 *g* for 5 min. After testing for the absence of interfering endogenous components, it was stored at −20°C.

On every analysis day, calibration samples for sulfadoxine were prepared by adding 100 μl of the freshly prepared drug solutions to 1 ml control plasma to obtain plasma drug concentrations in the range of 0.5–100 μg/ml. The anticoagulant used was 100 μl of a 5% solution of EDTA added to every 5 ml of blood.

2.3. SFC instrumentation

A JASCO-900 series (Japan Spectroscopic Co. Ltd., Hachioji, Tokyo, Japan) S.F. chromatograph was employed for the study. The apparatus was equipped with two pumps (PU-980) which could be adjusted for the flow of both CO_2 and the modifier from 0.001 to 10 ml/min. A Rheodyne model 7125 injection valve was used with an 20 μl external loop for introducing the sample into the analytical column. The temperature of the column was maintained using a JASCO-CO-965 series column oven. The outlet pressure was adjusted using a JASCO-880-81 back pressure regulator. Detection was done using a JASCO-UV-975 detector equipped with a 4 μl high pressure cell with a path length of 5 mm. A study of

the wavelength–absorbance relationship of sulfadoxine in the range of 210–350 nm obtained by injecting 20 μl of the extracted drug and measuring absorbances at different wavelengths showed that the drug exhibited a peak maximum at 265 nm in supercritical fluid CO_2. Hence this wavelength was chosen as the detection wavelength. The drug and the i.s. were chromatographed on a Machery–Nagel, Nucleosil-RP-C_{18} (250×4.6 mm) 10 μm packed-column. PC based Borwin-1.21 (JMBS Developments, Grenoble, France) chromatographic software was used for data integration. The optimum concentration of the mobile phase was found to 7.4% (v/v) of methanol in supercritical fluid CO_2. The CO_2 flow-rate was 2.5 ml/min with that of modifier being 0.2 ml/min. The outlet pressure was kept at 8.83 MPa and the temperature of the column and the mobile phase was 40°C. The injection volume was 20 μl. Sulfadoxine eluted at ~5.0 min and omeprazole at ~8.0 min. The total run time was about ~10 min.

2.4. Extraction from human blood plasma

Plasma samples were thawed at room temperature, vortex-mixed for 10 s and centrifuged at 1000 *g* for 5 min. A 1 ml aliquot of plasma (calibration standard, QC or unknown) was mixed with 100 μl of a methanol solution containing 150 μg/ml i.s., 100 μl methanol, 500 μl water and 100 μl buffer in 15 ml glass centrifuge tubes followed by addition of 5 ml dichloromethane. Extraction was performed on a rotating mixer for 20 min, followed by centrifugation at 3000 *g* for 20 min. The aqueous layer was discarded and 4 ml of the organic layer was transferred to another set of glass centrifuge tubes that had previously been rinsed with methanol. The samples were evaporated to dryness in a water bath at 45°C under a gentle stream of nitrogen. The residue was reconstituted with 0.2 ml of methanol and 20 μl of the solution were injected onto the SFC system under the above specified conditions.

2.5. Calibration and calculations

Eight calibration plasma samples covering the expected concentration range, Viz., 0.5, 1.0, 5.0, 10.0, 20.0, 50.0, 80.0 and 100.0 μg/ml of the drug were processed daily with QC and unknown samples, as described above. The calibration graph was obtained by plotting the peak area ratios (drug/i.s.) against the drug concentrations. A linear least squares fit analysis (weighting $1/X^2$) gave the best fit as

$$Y = (m \pm S_m) X + (b \pm S_b) \quad (1A)$$

$$Y = (0.2670 \pm 0.0005)X + (-0.0210 \pm 0.0246) \quad (1B)$$

where Y is the peak area ratio (drug/i.s.), X the drug concentration in μg/ml plasma, m the slope of the calibration graph, S_m the standard deviation of the slope, b the intercept on the Y-axis and S_b standard deviation of intercept. The regression factor was found to be 0.9999.

The standard deviation of residuals (S_{yx}) from the linear least squares fit was calculated to be 0.0544.

Every day the above calibration standards were sited at the beginning of each analytical batch. The regression lines established were used to calculate test analyte compositions by interpolation.

2.5.1. Quality control

To estimate the accuracy and precision of the method, quality control samples of drug-free plasma supplemented with 0.5, 1.5, 15, 40 and 80 μg/ml of sulfadoxine (with the requisite amounts of the i.s.) were prepared and stored at −20°C. These samples were analysed along with the test samples.

2.5.2. Clinical application

The assay method was successfully employed to measure the plasma concentrations of sulfadoxine in 12 healthy, male volunteers (age ~30 years) who had received an oral dose of 500 mg of sulfadoxine with 25 mg pyrimethamine. Blood samples were collected at 0, 1, 2, 4, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h after drug administration. The samples were frozen immediately after collection and stored at −20°C and analyzed by PCSFC within 7 weeks.

3. Results and discussion

3.1. Specificity and selectivity

The choice of the internal standard was affected by two requirements, quantitative recovery and a

suitable retention time in the chromatogram. Selectivity of the method was confirmed by analyzing 1.0 ml of drug-free plasma by the procedure described above but without any internal standard. Fig. 2A shows a typical chromatogram of the extract obtained by PCSFC while Fig. 2B shows the chromatogram of the extract from plasma spiked with sulfadoxine and omeprazole. Fig. 2C shows the chromatogram of prepared 1 h post dose plasma from a subject receiving 500 mg of sulfadoxine as a single

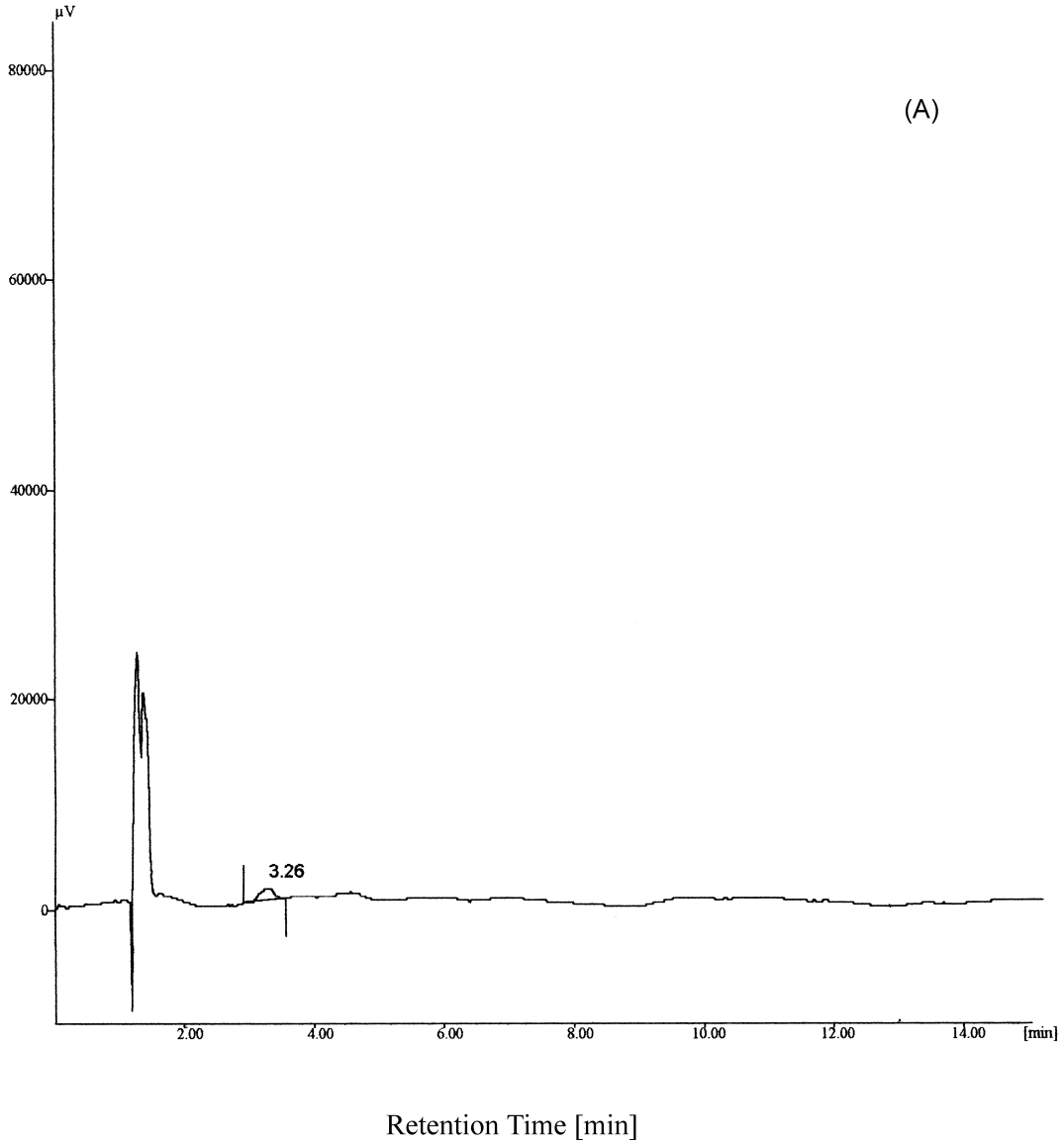

Fig. 2. (A) SFC Chromatogram of an extract from blank plasma. (B) SFC Chromatogram of an extract from blank plasma spiked with sulfadoxine (30 μg/ml, t_R = 5.09 min) and internal standard (omeprazole, 15 μg/ml, t_R = 8.09 min). (C) SFC Chromatogram of an extract from 1 h post dose (500 mg sulfadoxine) volunteer plasma sample; value found: 31.3 μg/ml of plasma. Peaks: Sulfadoxine = 5.26 min; I.S. = 8.28 min.

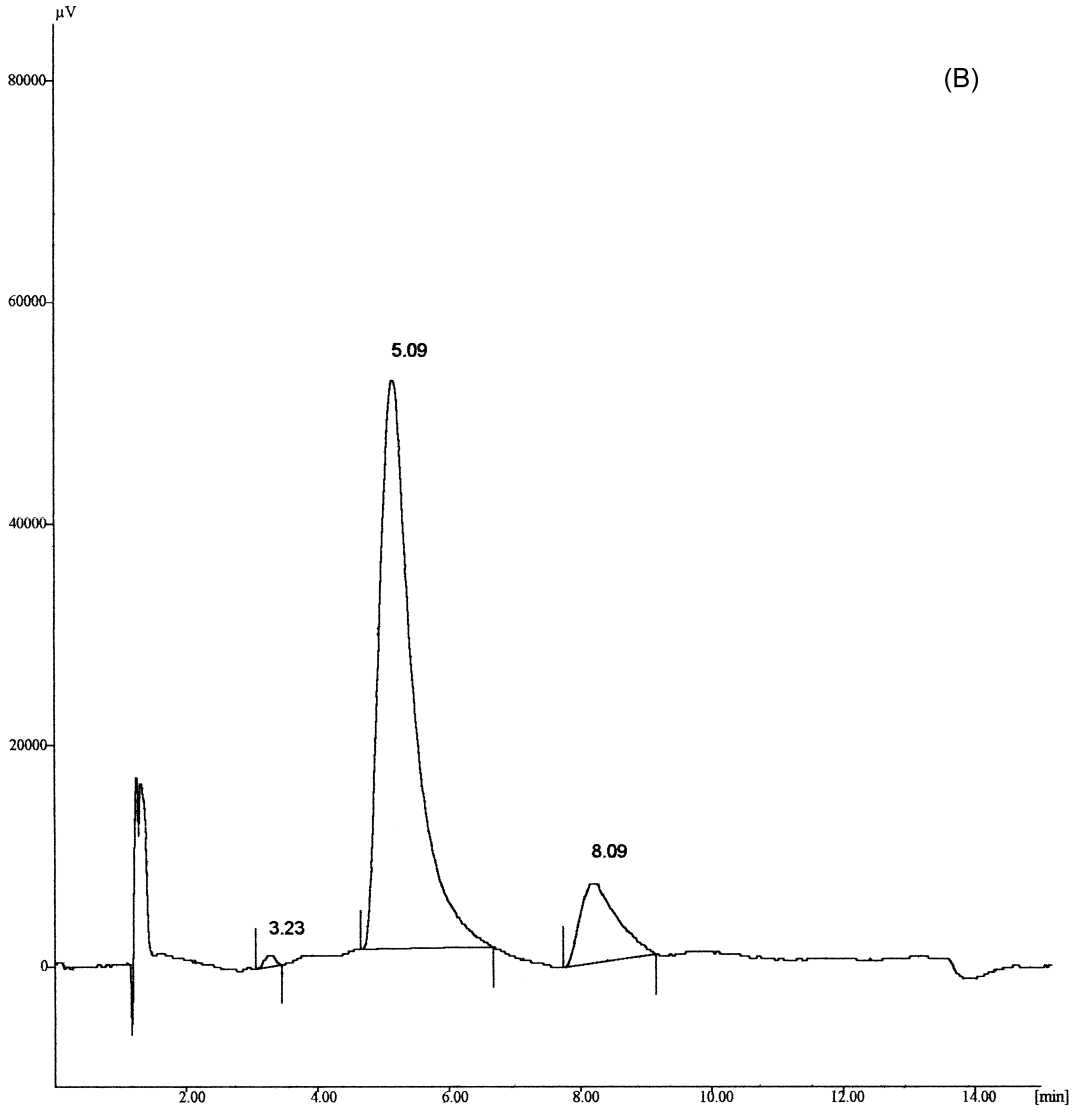

Fig. 2. (*continued*)

dose. Commonly used drugs, Viz.; fluconazole (t_R = 4.08 min), sulfaguanidine (t_R = 6.98 min), lansoprazole (t_R = 7.02 min), sulfamethoxazole (t_R = 12.39 min) and pyrimethamine, which is present in the formulation, were tested using this procedure for potential interference with sulfadoxine and omeprazole. The retention times of these drugs were found to be well separated from those of sulfadoxine and omeprazole. Pyrimethamine was not extracted.

3.2. Limit of quantification and limit of detection

The limit of quantification of the assay, defined as the minimum concentration that could be measured

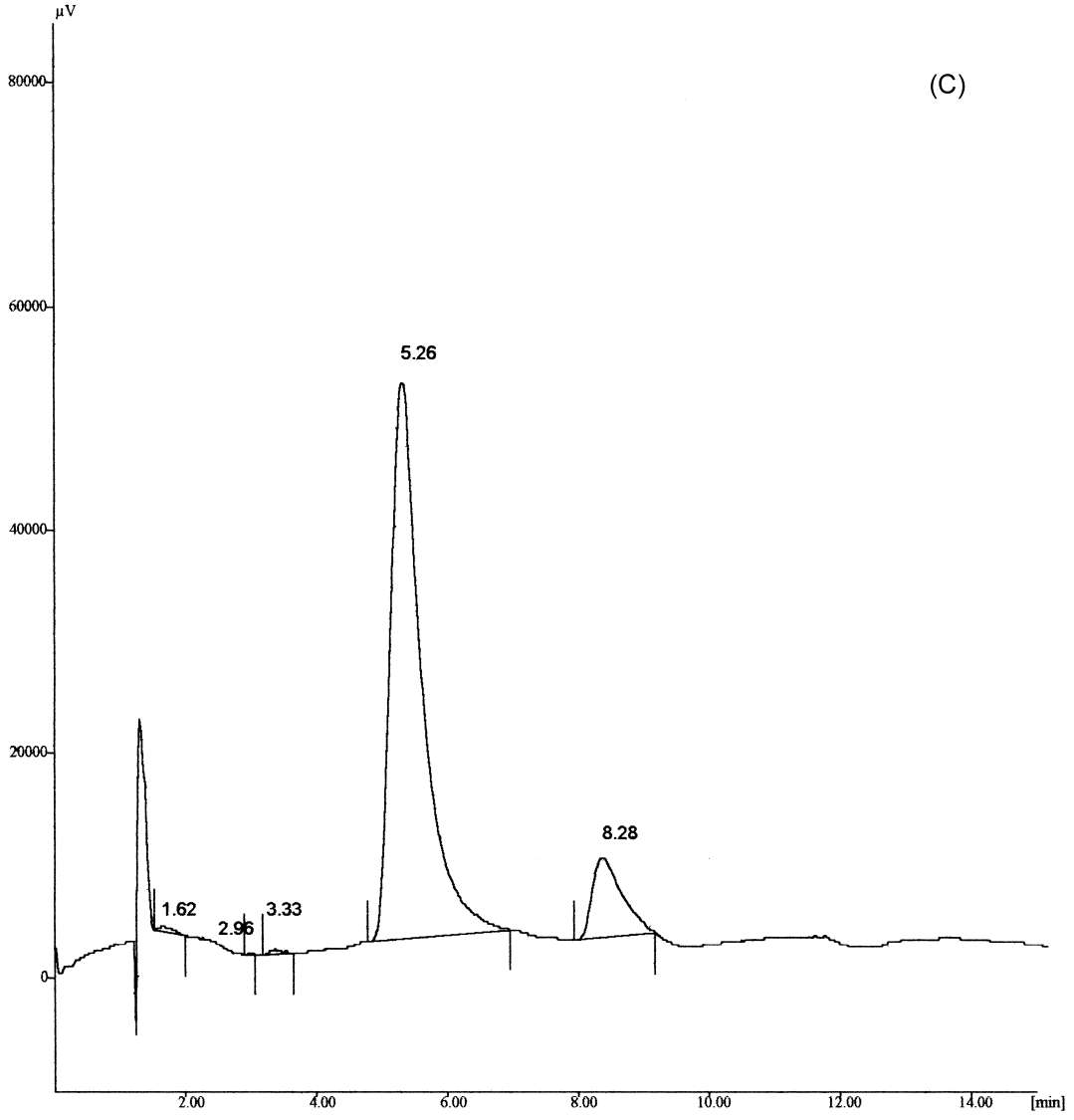

Fig. 2. (*continued*)

with a precision [coefficient of variation (C.V)] near about 10% was found to be 0.5 μg/ml.

The limit of detection, defined as the concentration giving a signal-to-noise ratio 3:1, was 0.15 μg/ml, using chromatographic and instrumental parameters defined above.

3.3. Recovery

Extraction recovery of the drug was determined by assaying spiked concentrations of the drug in plasma and methanolic solutions ($n=5$ each case) at three levels. Table 1 gives the results.

Table 1
Extraction recoveries of sulfadoxine from human plasma

Concentration (μg/ml)	Recovery (±SD)[a] (%)
0.5	92.9±1.4
10	95.7±1.3
80	96.0±0.2

[a] Each value is a mean of five determinations. Mean recovery = 94.9±2.0%.

Table 2
Accuracy and precision of the PCSFC method for the determination of sulfadoxine in spiked human plasma samples

Conc. added (μg/ml)	Conc. found[a] (μg/ml)	Error (%)	Accuracy (%)	RSD (%)
Intra-assay precision				
0.50	0.48	4.00	96.00	8.13
1.50	1.45	3.33	96.66	3.25
15.00	14.69	2.06	97.93	1.54
40.00	39.21	1.98	98.02	0.72
80.00	79.25	0.94	99.06	0.54
Inter-assay precision				
0.50	0.47	6.00	94.00	12.34
1.50	1.42	5.33	94.66	6.34
15.00	14.57	2.86	97.13	2.25
40.00	38.92	2.70	97.30	1.21
80.00	79.18	1.02	98.98	0.95

[a] Each value is a mean of five determinations ($n=5$).

3.4. Linearity

A linear correlation between the peak area ratios (drug/i.s.) and the concentration was found in the range 0.5–100 μg/ml. This wide range of concentrations necessitated the use of a weighting factor ($1/X^2$) in order to avoid division into sub-ranges. The correlation coefficient of determination was generally better than 0.9990 and the accuracy [(added − found)/added] for the daily studies was better than 6%.

3.5. Precision

The precision of the method was evaluated by replicate analysis of spiked quality control plasma samples. The intra-assay data were obtained by replicate analysis of QC plasma samples ($n=5$). The inter-assay data were obtained by analyzing the same QC plasma samples over a period of 7 weeks on days of analysis which came to forty times. The data shown in Table 2 demonstrate the good precision of the method over the concentration range investigated. The relative standard deviation varied from 0.5 to 8.1% for intra-assay precision and 0.9–12.3% for inter-assay precision. The results presented have been determined from the means of replicates per assay day.

3.6. Stability

For stability of sulfadoxine in plasma vials, containing known concentrations of the drug, at three levels, low, medium and high were stored at −20°C for a period of 115 days. The concentrations were chosen from the calibration range. Every day of

Table 3
Data on the usage and waste of organic solvents for estimation of sulfadoxine in human plasma using SFC and HPLC

	Packed column SFC/UV	Analytical scale HPLC/UV
Mobile phase	7.4% (v/v) of Methanol in SCF CO_2 at 2.5 ml/min	Acetonitrile: MeOH:1 *M* $HClO_4$: Water 23: 7: 0.6: 70 (1.0 ml/min)
Run time	10.0	13.0
Sample throughput, h^{-1}	6.0	4.6
Volume of mobile phase, h^{-1}	162 ml	60 ml
Volume of organic solvent, h^{-1}	12 ml	18.4 ml
Volume of disposable waste, h^{-1}	12 ml	60 ml

analysis of volunteer plasma one vial was taken and assayed together with the volunteer plasma samples. In these experiments sulfadoxine was stable during storage for 115 days.

3.7. Clinical results

The mean maximum peak plasma concentration (C_{max}) of sulfadoxine in twelve healthy male human volunteers obtained by PCSFC, following administration of 500 mg sulfadoxine in combination with 25 mg pyrimethamine as oral dose are shown in Fig. 3. The mean maximum plasma concentration of the drug (C_{max}) was 71.7 μg/ml at 4.0 h (T_{max}) after oral administration and the elimination half-life ($T_{1/2}$) was 155.8 h. The area under the curve (AUC) was 12394 μg h/ml.

The advantages of PCSFC became particularly striking when the solvent usage, sample throughput and waste disposal for SFC and HPLC were compared. Table 3 gives a comparison for these factors in the case of sulfadoxine plasma analysis. The

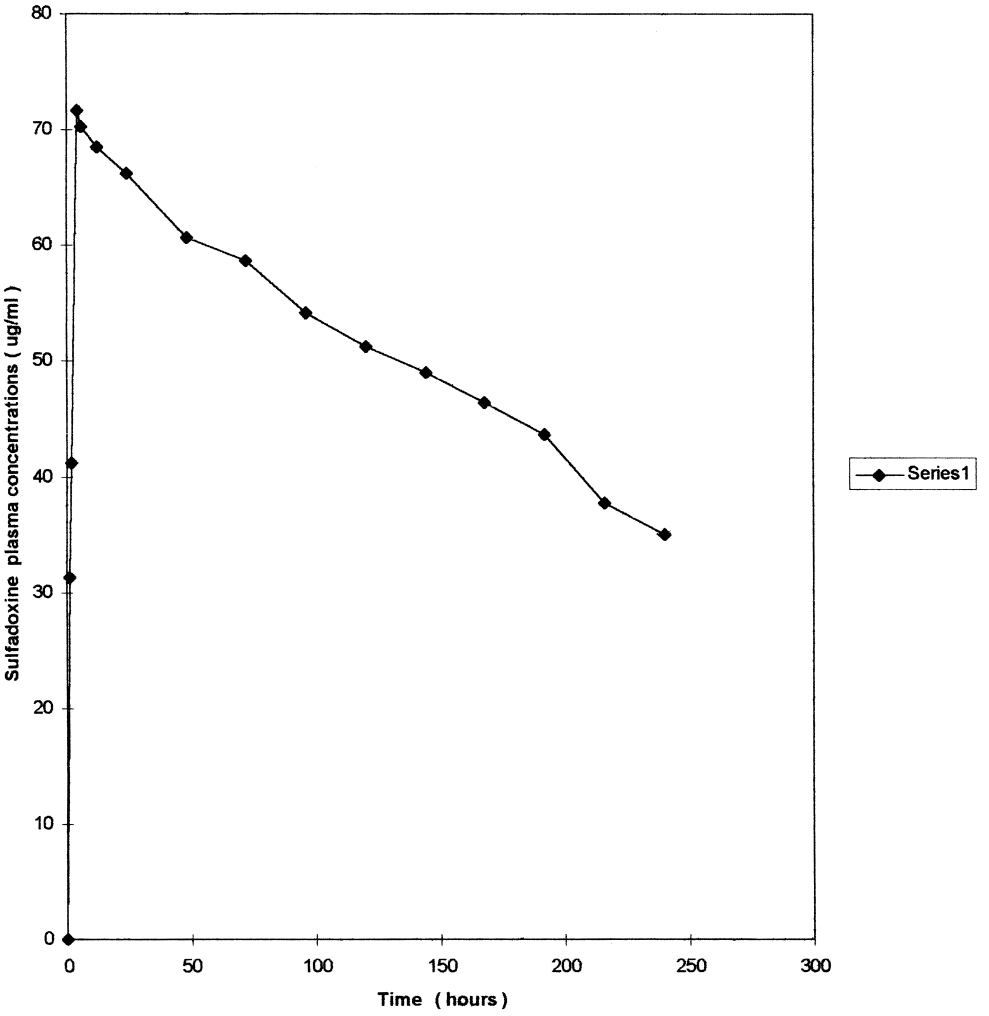

Fig. 3. Mean plasma concentrations of sulfadoxine–time in twelve healthy human subjects after the administration of a single 500 mg oral tablet in combination with 25 mg pyrimethamine.

conditions for HPLC were obtained from a published method [6].

4. Conclusions

The proposed method for the determination of sulfadoxine in human plasma has an advantage of high reproducibility and speed. As CO_2 is much cheaper than acetonitrile, the cost of analysis is significantly reduced as compared to the HPLC method. The technique offers a viable alternative to HPLC.

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References

[1] K. Parfeet (Ed.), Martindale – The complete drug reference, 32nd ed, The Pharmaceutical Press, London, 1999, p. 251, 436.
[2] C. Midskov, J. Chromatogr. 306 (1984) 388.
[3] H. Astier, C. Renard, V. Cheminel, O. Soares, C. Mounier, F. Peyron, J.F. Chaulet, J. Chromatogr. B 698 (1997) 217.
[4] Y. Bergqvist, S. Eckerbom, H. Larsson, M. Malekzadeh, J. Chromatogr. 571 (1991) 169.
[5] M. Edstein, J. Chromatogr. 305 (1984) 502.
[6] V.K. Dua, R. Sarin, V.P. Sharma, J. Pharm. Biomed. Anal. 12 (1994) 1317.
[7] B.R. Simmons, N.K. Jagota, J.T. Stewart, J. Pharm. Biomed. Anal. 13 (1995) 59.
[8] M.S. Smith, J. Oxford, M.B. Evans, J. Chromatogr. 683 (1994) 402.
[9] C.J. Bailey, R.J. Ruane, I.D. Wilson, J. Chromatogr. Sci. 32 (1994) 426.
[10] J.T.B. Strode, L.T. Taylor, A.L. Howard, M.A. Brooks, J. Pharm. Biomed. Anal. 12 (1994) 1003.
[11] D.L. Mount, G.D. Todd, V. Navaratnam, J. Chromatogr. 666 (1995) 183.
[12] Indian Pharmacopoeia, Publications and Informations Directorate (CSIR), Government of India, Ministry of Health and Family Welfare, New Delhi, India, (1996) pp. 533, 729.