

Short communication

Determination of sulphonamides in human urine by azo dye precolumn derivatization and micellar liquid chromatography

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Abstract

A high-performance liquid chromatographic method for the determination of sulphonamides in urine is reported. The drugs (sulphadiazine, sulphaguanidine, sulphamethizole, sulphamethoxazole, and sulphathiazole) were diazotized with nitrite and coupled with N-(1-naphthyl)ethylenediamine dihydrochloride in a sodium dodecyl sulphate (SDS) micellar medium. Separation of the sulphonamide azo dyes was performed on a C₁₈ column with a 0.05 M SDS–2.4% pentanol mobile phase, which permitted the direct injection of the urine samples. The limits of detection were in the 0.1–0.3 µg/ml range.

1. Introduction

Sulphonamides are used as antibacterial agents in medicine and veterinary practice. A benzene ring with a sulphonamido group and a primary amino group *para* to the sulphur side-chain are essential for antibacterial activity. Substitution in the amido group produces compounds varying in hydrophobicity, solubility, tissue distribution, and rate and mode of metabolism and excretion. The most active sulphonamides, such as sulphadiazine, sulphamethizole, sulphathiazole and sulphamethoxazole, have heterocyclic groups in the amido position [1].

The spectrophotometric determination of sulphonamides can adequately be performed by

formation of the azo dyes of the Bratton–Marshall reagent [N-(1-naphthyl)ethylenediamine dihydrochloride, NED] in a micellar medium of sodium dodecyl sulphate (SDS) [2,3]. However, some samples require chromatography or an other previous separation procedure [4,5], to avoid matrix effects and the interference of other drugs which could be present in the sample, or to resolve a mixture of sulphonamides. We have reported a liquid chromatographic procedure for the determination of sulphonamides in pharmaceuticals, where the compounds were separated with an SDS micellar eluent after precolumn derivatization to form the NED azo dyes [6]. In this work, a similar procedure for the direct analysis of urine samples is developed.

The determination of sulphonamides in biological samples usually requires the separation of

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proteins by means of laborious and time-consuming procedures of precipitation and extraction. The use of a surfactant in the mobile phase permits the direct injection of the samples in the chromatographic system [7–9]. The underivatized sulphonamides were poorly retained to allow direct injection of urine samples with SDS micellar mobile phases. As demonstrated in this work, the derivatization of sulphonamides increased their retention by decreasing the polarity of the solutes, and improved the signal-to-noise ratio and the resolution in the chromatograms. This allowed the adequate separation and detection of sulphonamides in urine.

2. Experimental

The sulphonamides studied (sulphadiazine, sulphaguanidine, sulphamethizole, sulphamethoxazole, and sulphathiazole) were present in pharmaceutical formulations commercialized in Spain. The reagents, apparatus and other experimental details are described elsewhere [6]. In the experimental procedure, 2 ml of the urine sample were introduced into a 25-ml volumetric flask, together with 10 ml of a 0.04 M SDS–0.15 M HCl solution and 1 ml of 0.1 M sodium nitrite. After 5 min, 1 ml of 0.3 M sulphamic acid was added, and the mixture was allowed to react for an additional 10 min. Finally, 0.5 ml of 0.03 M NED was added, the volume was completed to the mark with water and 20 μ l of the derivatized sample was injected into the liquid chromatograph. The azo dyes were formed immediately and were stable during several weeks, even when exposed to light and oxygen.

3. Results and discussion

The optimization of the separation of a mixture of compounds can be an easy task if an adequate description of the retention of each component is available. The model of retention used was [10]:

$$\frac{1}{k'} = A\mu + B\phi + C\mu\phi + D$$

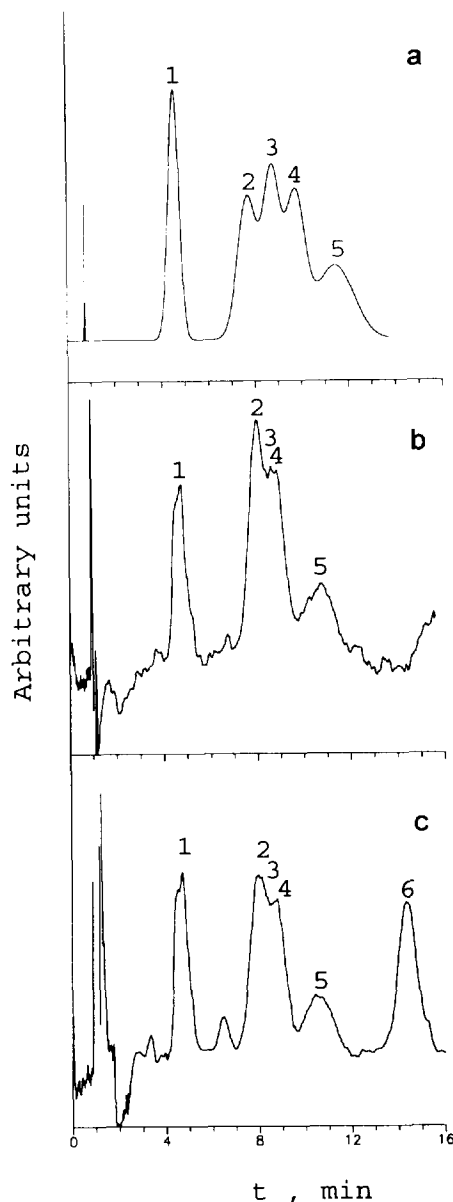


Fig. 1. (a) Predicted chromatogram for the mixture of the five sulphonamide azo dyes; (b) chromatogram of a water solution of the mixture; (c) chromatogram of a spiked urine sample. Mobile phase: 0.05 M SDS–2.4% pentanol. The concentration of each sulphonamide in (b) and (c) was 0.4 μ g/ml. Peaks: 1 = sulphamethizole; 2 = sulphaguanidine; 3 = sulphamethoxazole; 4 = sulphadiazine; 5 = sulphathiazole; 6 = endogenous compound in urine.

where k' is the capacity factor, μ is the molar concentration of surfactant, and ϕ is the volume fraction of alcohol. The model parameters were determined with five mobile phases: 0.05 M SDS–1.5% pentanol, 0.15 M SDS–1.5% pentanol, 0.05 M SDS–3.5% pentanol, 0.15 M SDS–3.5% pentanol, and 0.10 M SDS–2.5% pentanol. Good agreement between calculated and experimental capacity factors for the five sulphonamide azo dyes and five mobile phases, with a global error of 1.4%, was achieved. The optimization process was performed using an overlap criterion that considered the position and shape of the peaks [11]. The mobile phase giving the minimum overlap was 0.05 M SDS–2.4% pentanol. The predicted chromatogram for this mobile phase is shown in Fig. 1a for the mixture of the five azo dyes.

The high background at the beginning of the chromatogram of a urine sample, that appeared when detection was performed at 254 nm, was due to the presence of high-molecular-mass proteins and other endogenous compounds. Detection at a wavelength in the visible region eliminated this background signal. The chromatogram of a urine matrix treated with the diazotization and coupling reagents and detected at 488 nm, gave a peak at 13.5 min, which

corresponded to an unknown endogenous compound that probably formed an azo dye. However, this peak did not interfere with the determination of the sulphonamide azo dyes, which eluted at shorter retention times: sulphamethizole, 5.3 min; sulphaguanidine, 8.0 min; sulphamethoxazole, 8.5 min; sulphadiazine, 8.8 min; and sulphathiazole, 10.5 min.

Figs. 1b and 1c show the chromatograms of a mixture of the five sulphonamide azo dyes in water and in a spiked urine matrix, using a 0.05 M SDS–2.4% pentanol mobile phase. The chromatograms are similar, differing in the presence of the characteristic peak in the urine matrix. The peaks of the sulphadiazine and sulphamethoxazole azo dyes overlapped largely.

The calibration curves were obtained in the 1–20 $\mu\text{g/ml}$ range, with coefficients of linear regression $r > 0.99$ (Table 1). In urine, the slope of the calibration curves was 5–10% lower than in water. The limits of detection (LOD) were calculated from 8 replicates of 1 $\mu\text{g/ml}$ solutions of the sulphonamides. The reproducibility was lower in the urine matrix, as expected, but the LODs were similar for water and urine.

Following administration of a single oral dose of four sulphonamides to healthy volunteers, urinary excretion studies were performed. Chro-

Table 1

Analytical figures for the chromatographic determination of sulphonamides with precolumn NED azo dye derivatization, elution with a 0.05 M SDS/2.4% 1-pentanol mobile phase at pH 7, and detection at 488 nm

Compound	Matrix	Calibration line ^a		Reproducibility ^b ($n = 5$) (%)		LOD ($\mu\text{g/ml}$)
		Slope	Intercept	1 $\mu\text{g/ml}$	20 $\mu\text{g/ml}$	
Sulphamethizole	Water	16.4 \pm 0.1	-0.2 \pm 1.2	6.2	1.0	0.2
	Urine	15.3 \pm 0.1	0.5 \pm 2.1	9.2	3.2	0.3
Sulphaguanidine	Water	12.4 \pm 0.4	-1.4 \pm 4.8	7.8	3.8	0.1
	Urine	11.0 \pm 0.6	0.8 \pm 5.1	7.3	4.1	0.2
Sulphamethoxazole	Water	16.5 \pm 0.2	9.6 \pm 2.2	2.6	1.4	0.1
	Urine	15.8 \pm 0.3	8.2 \pm 3.2	5.1	3.9	0.1
Sulphadiazine	Water	16.4 \pm 0.1	0.7 \pm 1.6	7.0	1.0	0.02
	Urine	14.9 \pm 0.1	3.1 \pm 2.2	9.6	4.1	0.1
Sulphathiazole	Water	21.6 \pm 0.4	7.5 \pm 5.9	5.0	0.7	0.2
	Urine	20.3 \pm 0.5	8.5 \pm 3.2	3.2	4.3	0.2

^a Concentrations in $\mu\text{g/ml}$.

^b Reproducibility determined for the concentrations indicated.

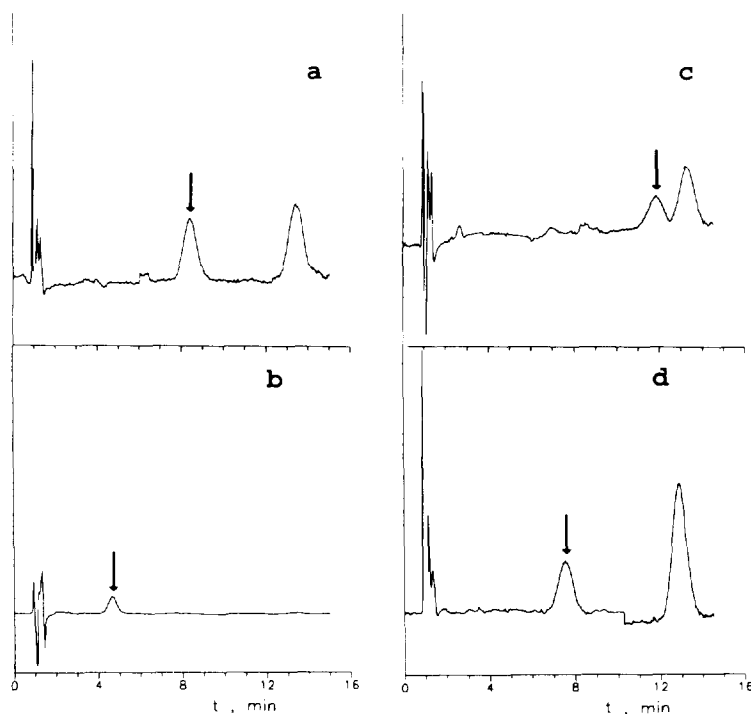


Fig. 2. Chromatograms of urine samples of healthy volunteers. The time after oral administration and the concentration of sulphonamide found in urine was: (a) 3 h, 7.7 $\mu\text{g/ml}$ sulphamethoxazole; (b) 7 h, 4 $\mu\text{g/ml}$ sulphamethizole; (c) 2 h, 7 $\mu\text{g/ml}$ sulphathiazole; (d) 14 h, 3 $\mu\text{g/ml}$ sulphaguanidine.

matograms of some urine samples are shown in Fig. 2. The recovered amounts of sulphonamides at several times after administration were similar to the data given in the literature [1]. The proposed method gathers the advantages of precolumn derivatization and chromatography with micellar mobile phases. Azo dye derivatization with NED is rapid, can be automated, and yields high molar absorptivities at large wavelengths which improves the signal-to-noise ratio when dealing with complex samples, such as urine. Detection after HPLC separation of the derivatives provided a selective and reliable method. Finally, the use of SDS micellar mobile phases on a C_{18} column is a versatile alternative with a very low polluting impact for the analytical laboratory dealing with drug development and quality control, and permits the direct injection of physiological samples.

Acknowledgements

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