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FLOW-INJECTION FLUORIMETRIC ANALYSIS OF SULFAMETHOXAZOLE IN PHARMACEUTICAL PREPARATIONS AND BIOLOGICAL FLUIDS

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Summary—A fluorimetric procedure for the determination of sulfamethoxazole using flow injection analysis is proposed. A two channel system is optimized for the fluorescent reaction with *o*-phthaldialdehyde and β-mercaptoethanol. The detection limit is 0.007 μg/ml. No fluorescence is generated by the metabolite *N*-acetylated sulfamethoxazole. The method is applied to the determination of sulfamethoxazole in pharmaceuticals, urine and bovine serum samples without matrix interference problems.

Sulphonamides were the first chemotherapeutic agents effective for the treatment of the bacterial infections in man.[1] They have been widely studied and their pharmacokinetic parameters were established using different methods for drug determination in biological fluids. Sulfamethoxazole is the most useful sulphonamide for the control of bacterial diseases. The main fraction of the drug is excreted by the urine, and the half-life depends on the renal function. The periodic determination of the plasmatic concentration of the free drug is advisable when patients are treated with sulphonamides. The main metabolic derivative is N^4-acetylated sulfamethoxazole. This acetylated product has a toxic effect but no antibacterial activity. For routine analysis, spectrophotometric methods based on the Bratton–Marshall reaction[2] and chromatographic techniques, especially HPLC,[3] have been used. A fluorimetric method involving diazotization of the amine and oxidation of the resulting azo-compound to a fluorescent triazole has been proposed by Narita *et al.*[4] Flow injection analysis (FIA) with spectrophotometric detection has also been reported.[5–7] FIA–fluorimetric methods could be of practical use since they combine the advantages of easy automatization with a very sensitive detection technique. For the fluorescent detection of amines and amino acids, the reagent *o*-phthaldialdehyde (OPA) is currently used in combination with a thiol compound, usually β-mercaptoethanol (ME).[8–10] This reaction was studied with the stopped-flow technique by Trepman and Chen.[11] However, it has not been applied to the determination of sulphonamides.

In the present study, a sensitive, simple and rapid procedure for the determination of sulfamethoxazole using its reaction with *o*-phthaldialdehyde and β-mercaptoethanol is reported. Flow injection analysis with fluorimetric detection is used. The method has been applied to the analysis of the drug in pharmaceutical preparations and biological fluids, such as urine and serum, without pretreatment of the samples. N-Acetylated sulfamethoxazole did not produce any fluorescent product and, consequently, there was no interference from metabolites when biological samples were analyzed.

EXPERIMENTAL

Instrumentation

A Kontron SFM 25 fluorescence detector was used at wavelengths of 302/412 nm excitation/emission. A Philips PM 8100 recorder and a Colora thermostat were also used. The flow injection system consisted of a Gilson Minipuls HP4 peristaltic pump, an Omnifit injection valve, a Hellma 176.052-QS fluorimetric flow cell, 0.5 mm i.d. PTFE tubing and various end fittings and connectors (Omnifit).

Reagents

High quality water obtained using a Milli-Q system (Millipore) was used throughout. *o*-Phthaldialdehyde (Fluka) and β-mercaptoethanol (Sigma) 0.01*M* solutions were prepared by dis-

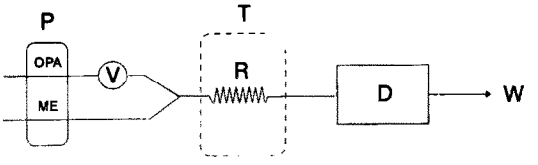

Fig. 1. Flow-injection manifold for the analysis of sulfamethoxazole. (P) Peristaltic pump, total flow-rate 1 ml/min; (V) injection valve, sample-loop 200 μl; (R) reactor coil, 2.5 m long and 0.5 mm i.d.; (T) thermostat at 40°C; (D) fluorimeter 302/412 nm excitation/emission; (W) waste.

solving the products in 2% ethanol and 0.7*M* phosphoric acid and kept in dark bottles at 4°C. Sulfamethoxazole (Sigma) 500 μg/ml stock solution was prepared in ethanol and also kept at 4°C; working standard solutions were prepared by dilution with water just before use.

Analytical procedures

The flow manifold is shown in Fig. 1. A two-channel system was used. *o*-Phthaldialdehyde 0.01*M* in 0.7*M* phosphoric acid flowed into one channel in which sulfamethoxazole was injected. This solution merged with a stream of 0.01*M* β-mercaptoethanol in 0.7*M* phosphoric acid at a PTFE T-piece. The resulting solution flowed through a 40°C thermostated 2.5 m reactor coil and into the flow cell to record the fluorescence intensity. Both carrier streams were pumped at the same flow rate by means of a peristaltic pump with a total flow rate of 1.0 ml/min. A T-piece was chosen as the mixing device to obtain the most effective and rapid mixing of the reagents. Samples were injected (200 μl loop) into the OPA stream. The fluorescence increase was recorded and the peak heights measured. Calibration graphs were obtained by plotting peak heights against the sulfamethoxazole concentration.

For determination of the drug in pharmaceutical preparations, each commercial sample (5 ml of syrup, the total capsule or tablet, or 0.25 g of the powder dissolved in 50 ml of 1:1 ethanol:water mixture) was dissolved with ethanol up to 250 ml in a calibrated flask. An aliquot was filtered through a 0.2 μm nylon Millipore chromatographic filter and analyzed by the FIA method. For the studies of the sulfamethoxazole recovery from urine and serum samples, the only pre-treatment required was dilution with water and filtration.

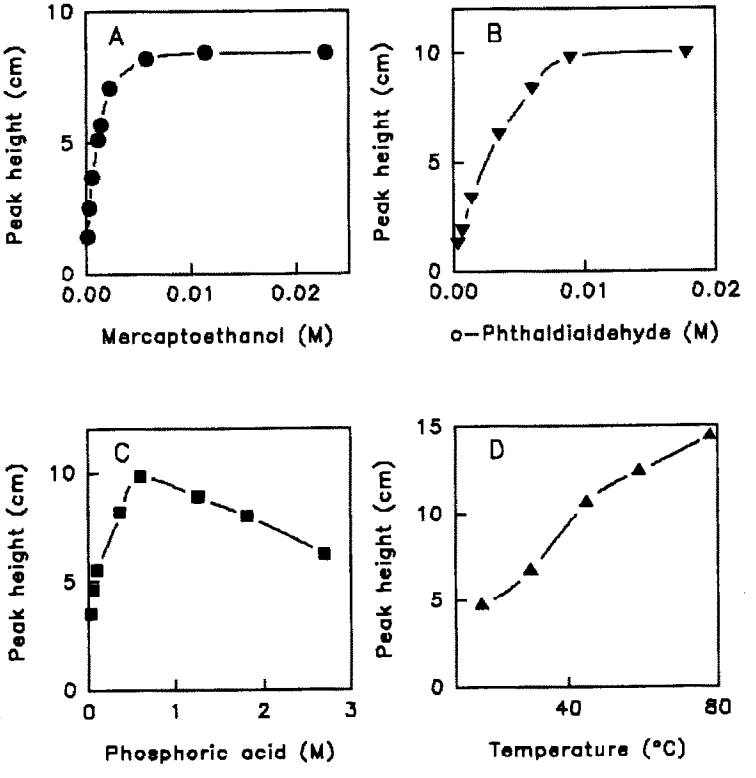

Fig. 2. Influence of the variation of experimental conditions on the fluorescence. (A) β-Mercaptoethanol, (B) *o*-phthaldialdehyde, (C) phosphoric acid and (D) temperature.

Preparation of the N-acetylated sulfamethoxazole derivative

Acetylation of the primary amine was carried out with acetic anhydride following a described procedure.[12] Recrystallization was performed from an ethanol–water mixture. The synthetized compound was identified using elemental analysis, mass spectrometry and nuclear magnetic resonance spectrometry.

RESULTS AND DISCUSSION

The mechanism of the reaction of *o*-phthaldialdehyde and β-mercaptoethanol with primary amines has been previously described.[13] The reaction between OPA, ME and sulfamethoxazole is slow. Since the development of the fluorescent product is a strongly time-dependent process, the essential performances of flow injection methodology permitted a time-controlled dispersion of the sample and, consequently, a reproducible analytical signal.

Optimization of the experimental conditions

The influence of the ME concentration was studied between 10^{-4} and $0.023M$ by injecting 1.2 μg/ml of sulfamethoxazole into the OPA stream. Figure 2A shows that fluorescence quickly increased with ME up to a concentration of $6 \times 10^{-3}M$. To obtain a signal in the plateau, a $0.01M$ value was chosen as optimal. Figure 2B illustrates a similar variation of the signal with the OPA concentration in the 3×10^{-4}–$0.018M$ range, from which $0.01M$ was selected as optimum. The pH of the medium had a noticeable effect on the reaction. The OPA–ME reaction with aliphatic amines is usually carried out under initial alkaline conditions with subsequent acidification to stabilize the adduct obtained and to increase the fluorescence. We studied the reaction of OPA–ME with SM using sodium hydroxide in the initial alcalinization step before acidification with phosphoric acid at different times. Since the reaction was slow, an attempt was made to eliminate the alkaline step, similar results being obtained. The effect of the acidity was studied with phosphoric, acetic and hydrochloric acids. Phosphoric acid generated intense fluorescence signals; therefore, the optimal concentration was studied by adding different amounts of the acid to both the OPA and ME streams. Figure 2C demonstrates that the fluorescence reached a maximum at *ca.* 0.6–0.8M acid concentration and then decreased when higher concentrations were employed. No blank signals appeared at any of the acid concentrations examined. Consequently, $0.7M$ phosphoric acid was added to the reagents. The different behaviour of SM and the aliphatic primary amines usually determined using this reaction may be due to the aromatic structure of the SM. This could produce an electronic shift in the amine group, thus allowing the binding of the primary amine with the OPA–ME adduct directly in an acidic medium.

Influence of the FIA variables

The influence of the reaction coil length was studied in the 0.3–4.0 m range using the previously optimized experimental conditions. As expected (Fig. 3), the fluorescence increased with the length of the reactor. Thus, an optimal value of 2.5 m was selected. The effect of the sample loop size was examined between 60 and 500 μl and linearity was observed up to approximately 200 μl, this being the loop size selected (curve B). Variation of the total flow rate between 0.6 and 2.7 ml/min produced a decrease in the fluorescence as the flow rate increased (curve C). When the residence time of the sample in the mixing coil was small, the extent of the reaction decreased considerably. A 1 ml/min value (0.5 ml/min for each reagent) was

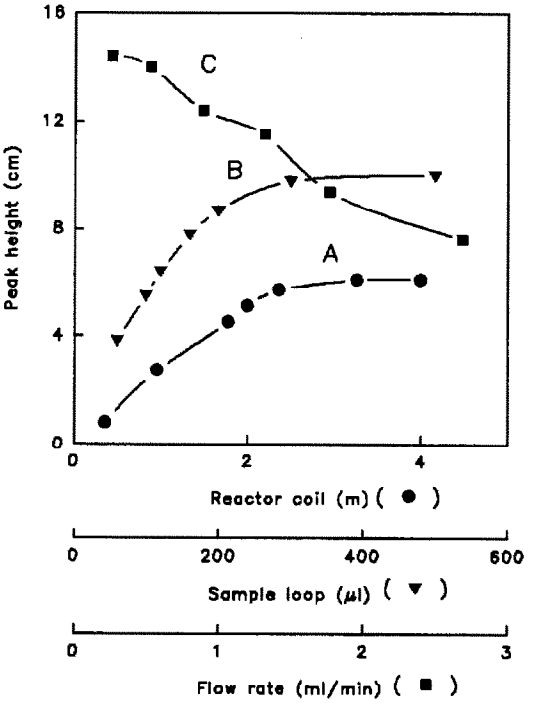

Fig. 3. Effect of FIA variables. (A) Reactor coil length, (B) sample loop size and (C) flow rate.

selected as a compromise between a good signal and an adequate sampling frequency.

Influence of the temperature

Formation of the fluorescent product occurs slowly; therefore, heating of the reactor coil to increase the reaction rate was examined. Figure 2D shows the results obtained when the reactor was immersed in a water bath at temperatures between 17 and 80°C. The signal increased with an increase in temperature. However, a 40°C temperature was selected to simplify the experimental work and avoid bubble formation.

Calibration, interferences and applications

Figure 4 shows the FIA peaks obtained for the calibration of sulfamethoxazole. Linearity was obtained between 0.01 and 2.5 μg/ml. The detection limit calculated on the basis of 3σ was 0.007 μg/ml. The precision of the procedure was obtained from the relative standard deviations (RSD) calculated for 10 replicate determinations from three sulfamethoxazole concentrations. RSD values were ± 3.5, ± 2.2 and $\pm 2.1\%$ for 0.01, 0.05 and 1.5 μg/ml, respectively.

Interferences caused by the common tablet fillers were studied by injecting solutions containing sulfamethoxazole (0.2 μg/ml) and different amounts of the other compounds. No interferences were found for fructose, lactose, galactose, glucose, saccharose, maltose, lysine, caffeine, ascorbic acid, acetylsalicylic acid, paracetamol, citrate and starch at ratios [interferent]/[sulfamethoxazole] up to 100/1. Higher concentrations were not assayed. Saccharin was tolerated up to a 10/1 ratio. The tolerance limit was taken as the concentration causing an error of no more than $\pm 3\%$ in sulfamethoxazole recovery. In pharmaceuticals, trimethoprim is usually combined with sulfamethoxazole and so its effect was assayed. The optimal relation between the concentrations of the two agents for synergy is 20:1 sulfamethoxazole/trimethoprim. It was found that no interference appeared when trimethoprim is present in a 100-fold excess. The reactivities of histidine and histamine were also examined and no interferences in SM determination were detected. The high selectivity of the reaction was achieved because these amines did not react with OPA and ME in acidic medium. Interferences by other sulphonamides were also tested. Sulfanilamide, sulfaguanidine, sulfadiazine, sulfathiazole and sulfapyridine also yielded fluorescence products. Both differences in the reaction rate and the fluorescent quantum yield due to these species, caused different slopes of the calibration graphs for each sulphonamide.

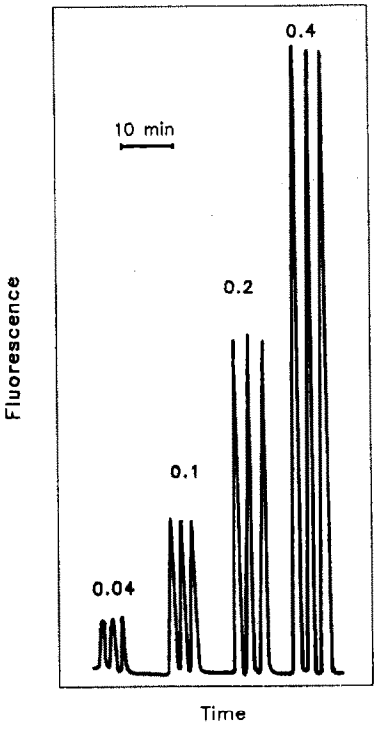

Fig. 4. FIA peaks for calibration of sulfamethoxazole. Numbers on the peaks correspond to μg/ml of the drug.

To assess the utility of the proposed method, several pharmaceutical preparations in different physical forms were analysed. Results are included in Table 1. All values are in good statistical agreement with the values supplied by the manufacturers.

The usual dose in the adult is 800 mg of sulfamethoxazole every 12 hr for 10–14 days. Between 70 and 100% of the oral dose is absorbed and 25–50% is excreted by urine within 24 hr. The drug can be detected in the urine 30 min after ingestion.[1] Since the main acetylated metabolite did not react, the procedure is highly selective for the drug. It was, therefore, applied to the determination of the drug in urine obtained from a healthy volunteer. Different volumes of urine were diluted to 50 ml and centrifugated. A 2 ml aliquot was again diluted to 250 ml with water. The method of standard additions was used to investigate the possibility of interference caused by the matrix. Each graph was constructed from four points and each point was measured three times. Table 2 shows that the slopes of the aqueous standards and standard additions graphs were similar,

Table 1. Determination of sulfamethoxazole in pharmaceutical preparations

Product (Laboratory)	Sulfamethoxazole	
	Reported	Found*
Septrim (Wellcome)	40 *mg/ml* suspension	39.0 ± 2.8
Bronco-bactifor (Andrómaco)	400 *mg*/7.5 *ml* suspension	412 ± 12.7
Pulmo-Menal (Alacan)	400 *mg*/7.5 *ml* suspension	398 ± 10.5
Salvatrim (Salvat)	400 *mg*/capsule	405 ± 4.6
Eduprim (Alonga)	400 *mg*/tablet	407 ± 6.9
Soltrim (Almirall)	800 *mg*†/vial	802 ± 21.0

*Mean ± standard deviation (SD) ($N = 5$).
†As lysinate of sulfamethoxazole ultrasoluble.

Table 2. Slopes of the standard additions calibration graphs

Sample	Slope (mean ± SD)
Aqueous standard	52.76 ± 1.56
Urine	49.09 ± 1.26
Serum	50.21 ± 1.01

thus confirming that no interference from the matrix urine was present and that the method allows aqueous standards to be used for calibration. The results for the recovery of the drug from urine samples are presented in Table 3. The method was also applied to bovine serum samples. Different volumes of serum were diluted to 10 ml and a 2 ml aliquot was again diluted to 250 ml with water. Table 2 shows that no matrix interference existed and good recoveries were also obtained (Table 3). It has been reported that the maximum concentration in human plasma is generally reached 4 hr after an oral dose and the mean life is about 9 hr. When 800 mg are administered two times per day, the plasmatic concentration of the drug is approximately 40 μg/ml.[1] Therefore, it is clear that the procedure is sensitive enough to be applied to the determination of the drug in biological fluids, even after the samples are diluted, thus minimizing possible problems due to this organic matrix.

CONCLUSIONS

A comparison of the proposed FIA–fluorimetric method with other existing methods shows advantages as regards simplicity, sensitivity and selectivity. The simplicity of sample preparation and the automatic control of time using the FIA system means a less expensive and more versatile system with considerably reduced analysis times compared with reported manual methods. The exact control of time allows a better accuracy and reproducibility. The sensitivity is higher than that obtained using spectrophotometric procedures and the on-line dilution prevents matrix effects. Most of the reported methods are not selective because of the interference of other primary amines. However, no interferences of histidine and histamine are observed with the proposed method. Linearity, precision and recovery are also satisfactory. In summary, the method described here is simple, highly sensitive, selective and useful for the routine quantitative analysis of sulfamethoxazole in pharmaceuticals and biological samples with minimal sample conditioning.

Table 3. Recovery results for sulfamethoxazole in spiked urine and serum samples

Urine				Serum			
Sample volume (*ml*)*	Sulfamethoxazole (μg/*ml*) Added	Found	Recovery (%)	Sample volume (*ml*)†	Sulfamethoxazole (μg/*ml*) Added	Found	Recovery (%)
2	0.082	0.084	102.4	0.4	0.080	0.072	90.0
2	0.164	0.169	103.0	0.4	0.162	0.160	98.8
2	0.246	0.253	102.8	0.4	0.242	0.248	102.5
5	0.082	0.083	101.2	1	0.080	0.082	102.5
5	0.164	0.163	99.4	1	0.162	0.164	106.2
5	0.246	0.249	101.2	1	0.242	0.236	97.5
10	0.082	0.080	97.6	2	0.080	0.084	105.0
10	0.164	0.157	95.7	2	0.162	0.166	102.5
10	0.246	0.239	97.2	2	0.242	0.248	102.5

*Diluted to 50 *ml* with water.
†Diluted to 10 *ml* with water.

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