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Note

High-performance liquid chromatographic method for determination of sulfapyridine in human saliva using post-column, in-line derivatization with fluorescamine

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Sulfasalazine (salicylazosulfapyridine), a drug used to treat ulcerative colitis [1—4] is itself poorly absorbed in the human gastrointestinal tract [5]. It is reduced by bacteria in the colon and cecum to sulfapyridine and 5-amino-salicylic acid [6, 7] which are absorbed.

Numerous analytical methods for analysis of sulfapyridine and the other metabolites of sulfasalazine have been reported. These methods include the Bratton and Marshall-based colorimetric method [8], high-performance liquid chromatography (HPLC) [9—14] and gas—liquid chromatography [15, 16]. These methods employ lengthy extraction procedures, and for the most part suffer from a lack of sensitivity and specificity.

In this paper, an HPLC method with post-column, in-line derivatization of sulfapyridine with fluorescamine followed by measurement of the generated fluorophore with a fluorometric detector is described. Using this method, an increase in sensitivity above that achievable using existing methods was demonstrated. The extraction process is a single-step, rapid process that allows analysis of large numbers of samples quickly.

The use of fluorescamine in post-column, in-line derivatization has presented problems due to the instability of the fluorescamine solution. In this paper, a solution is described in which fluorescamine is stable for up to 48 h.

EXPERIMENTAL

Apparatus

The liquid chromatographic system was assembled from two Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery systems, a Waters Assoc.

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Model 710 B WISP automatic sample processor, a 12.5 cm × 4.6 mm I.D. stainless-steel column packed with RP-18 (5 μm; Brownlee Labs., Santa Clara, CA, U.S.A.), a Schoeffel Instrument (Westwood, NJ, U.S.A.) Model FS 970 spectrofluorometer operated at $\lambda_{ex.}$ = 395 nm and $\lambda_{em.}$ = 470 nm, coiled Polytef tubing (4.8 m × 0.7 mm I.D.) which served as a post-column, in-line reactor, a Thermonix (B. Braun) Model 1420 water bath (60°C) in which the Polytef tubing was immersed, and an Altech (Arlington Heights, IL, U.S.A.) T-fitting which served to connect the reactor, one pump, and the column. The liquid chromatograph was connected to a Spectra Physics (Santa Clara, CA, U.S.A.) Model 4100 integrator-calculator.

Reagents

Sulfapyridine was obtained from Applied Science Labs. (State College, PA, U.S.A.), sulfadiazine from Sigma (St. Louis, MO, U.S.A.), and Fluram from Pierce (Rockford, IL, U.S.A.). The solvents methanol and acetonitrile were analytical-reagent grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). 1-Hexanesulfonate as the sodium salt was from Regis (Morton Grove, IL, U.S.A.), while triethylamine was from Pierce. Reagent grade 2-mercaptoethanol was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and 5-aminosalicylic acid was from Aldrich (Milwaukee, WI, U.S.A.). Sulfasalazine tablets were obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.).

Solutions

The mobile phase was composed of 0.05 *M* $NaHPO_4$, 0.01 *M* 1-hexanesulfonate sodium salt, 0.0072 *M* triethylamine, pH 3.0 (using phosphoric acid), and 15% methanol. The volumetric flow-rate of the mobile phase was 1.0 ml/min.

The Fluram solution introduced into the mobile phase, post-column, was prepared as follows: Fluram (400 mg) was dissolved in methanol (250 ml) followed by addition of 2-mercaptoethanol (1 ml), and mobile phase (250 ml). Volumetric flow-rate of this solution was 0.3 ml/min.

Stock solutions of sulfapyridine and internal standard, sulfadiazine, 1 mg/ml in methanol were prepared and stored at 4°C. A standard curve was generated by spiking blank saliva samples (1 ml) with varying amounts of sulfapyridine and a constant amount of internal standard. Microliter aliquots of the stock solutions or a 1:10 dilution of the stock solutions in water were added to the saliva. The internal standard concentration in saliva was 148 ng/ml. The sulfapyridine concentration range in saliva was 20.0—497.7 ng/ml. These samples were treated according to the extraction procedure described.

Analytical procedure

Acetonitrile (1 ml), solid potassium carbonate (approximately 400 mg), and internal standard, sulfadiazine (148 ng/ml saliva) were added to saliva samples (1 ml) in 15-ml glass stoppered centrifuge tubes. The tubes were then shaken on a vortex mixer for 1 min. After centrifugation ($\geqslant$1000 *g*) for 10 min, the upper acetonitrile layer was transferred to a second centrifuge tube followed by evaporation to dryness under nitrogen in a 60°C water bath.

The residues were then dissolved in mobile phase (200 μl), and mixed in the vortex mixer. Aliquots (40 μl) of these solutions were then injected into the chromatographic system.

RESULTS AND DISCUSSION

Plots of the peak area ratios (sulfapyridine to internal standard) against the respective sulfapyridine concentrations were linear. Typical standard curves for sulfapyridine in saliva had correlation coefficients of 0.9998, over a range of 20 to 498 ng/ml. The coefficients of variation for five replicate assays at 20.0, 49.8, 97.4, 248.9 and 497.7 ng/ml of saliva were ±6.6, ±3.7, ±8.4, ±6.4 and ±6.4%, respectively. A typical chromatogram for a saliva extract is shown in Fig. 1.

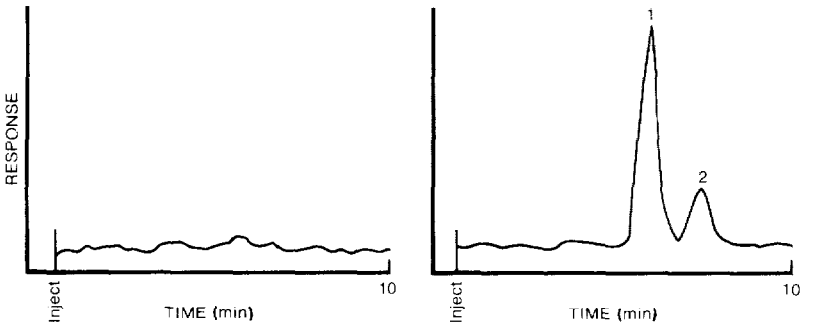

Fig. 1. Chromatograms of an analysis of a saliva blank (left) and of a saliva sample with added internal standard and sulfapyridine (right) carried through the procedure (148 and 20 ng/ml, respectively). Peaks: 1 = internal standard, sulfadiazine (retention time = 5.66 min); 2 = sulfapyridine (retention time = 7.32 min).

The achievable detection limit for sulfapyridine in saliva using the method described is 5 ng/ml (1 ml of saliva volume). The method is more sensitive than existing methods utilizing HPLC [9—14]. The high sensitivity of the method is due to the specificity of the fluorescence detection step for sulfapyridine relative to the low biological background. The extraction procedure is rapid, allowing analysis of up to 100 samples per day.

Acetonitrile has been reported to be preferable to methanol both as an organic modifier in the mobile phase and as a solvent for the fluorescamine reagent. This is presumably due to an increase in reaction time between fluorescamine and primary amines caused by a reversible hydrolysis of fluorescamine by alcohols [17, 18]. In addition, hydrolysis of fluorescamine has been reported to result in the formation of a yellow (fluorescent) end-product [18, 19], causing a rising baseline during the chromatographic step. We have found that the addition of 2-mercaptoethanol to the Fluram solution prevents baseline drift for up to 48 h. After this addition, there were no differences between acetonitrile and methanol in terms of obtainable detection limits for sulfapyridine or stability problems of the Fluram solution for up to 48 h.

The effect of pH on fluorescence intensity was studied. There was little dif-

ference in intensity between pH 2.5 and 4.0, except that at pH 4.0 the Fluram solution became yellow at a faster rate. At pH 7.3, the Fluram solution turned yellow within minutes of being made up, and neither sulfapyridine nor sulfadiazine could be detected using this solution in the chromatographic system. Sulfanilamides are reported to react most favorably at pH 3.0—4.5, and have maximum fluorescence intensity at pH 3.0—4.0 [20].

No interference to either sulfapyridine or sulfadiazine was found from the following compounds which are either primary amines or naturally fluorescent; amphetamine, 2-amino-3-phenyl-1-propanol, furosamide, salicylic acid, 5-aminosalicylic acid, sulfasalazine, N-acetylsulfapyridine, viloxazine, levallorphan, metoprolol and riboflavin.

To demonstrate the usefulness of this method, results from the analysis of saliva samples obtained from a healthy volunteer who had ingested a 500-mg Azulfidine tablet (Pharmacia) are shown in Table I.

TABLE I

SALIVA CONCENTRATIONS OF SULFAPYRIDINE IN A HUMAN AFTER A SINGLE 500-mg ORAL DOSE OF AZULFIDINE

One subject was studied.

Time after dose (h)	Saliva sulfapyridine (ng/ml as free base)
4.0	<20
5.0	<20
6.0	<20
7.0	25
7.5	208
8.0	423
8.5	682
9.0	955
10.5	1376
12.5	102
21.5	212

The method has been used to analyze sulfapyridine in over 1500 saliva samples. No deterioration of column performance or of the assay as a whole was observed.

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