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Note

Quantitative thin-layer chromatography of trimethoprim and tetroxoprim using fluorescence densitometry

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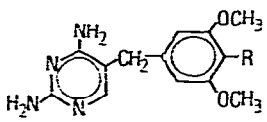
The dihydrofolic acid reductase inhibitors trimethoprim (TMP) and tetroxoprim (TXP) (Fig. 1) in combination with various sulfonamides are used extensively in human and veterinary medicine in the treatment of a variety of bacterial infections [1, 2].



TMP: R = OCH_3

TXP: R = $OCH_2CH_2OCH_3$

Fig. 1. Chemical structures of trimethoprim (TMP) and tetroxoprim (TXP).

A number of analytical methods for the quantitation of TMP in biological fluids have been described including, among others, gas—liquid chromatography [3] and high-performance liquid chromatography (HPLC) [4—6]. For TXP an HPLC assay method has been employed [7]. For TMP also two thin-layer chromatographic (TLC) assay methods have been described based on either absorption densitometry [8] or fluorescence densitometry [9]. The fluorescence that was measured in the latter method originated from a product (or products) that developed on the thin layer on exposure (for at least 24 h) to light.

In this present paper a quantitative TLC assay for TMP and for TXP is

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described which is based on the densitometric measurement of the fluorescence that develops within 10 min after in situ treatment with nitric acid.

MATERIALS AND METHODS

Sample preparation

Plasma samples (1 ml) containing either TMP or TXP were made alkaline with 0.5 ml of 1 *N* sodium hydroxide. Extraction was performed with 7 ml of a dichloromethane—hexane (1:1, v/v) mixture. An aliquot (6 ml) of the organic layer was transferred to a tapered centrifuge tube and evaporated to dryness at 40°C under a stream of nitrogen. The sides of the tube were rinsed down with 0.5 ml of chloroform. The final residue was taken up in 50 μl of chloroform. The tubes were centrifuged shortly in the cold to force the fluid into the tip. The stoppered tubes were kept in the cold (4°C) until the samples were spotted.

Urine samples were handled in the same way or, depending on the concentration, were spotted directly.

Chromatography

Aliquots of the samples (usually 10—20 μl of the final extracts, or 2—5 μl of undiluted urine) were applied to a silanized silica gel plate (20 × 20 cm, 0.25 mm thick; Merck, Darmstadt, G.F.R.), at 1 cm distance from each other. Chromatography was carried out according to the ascending technique in a filter-paper lined tank. The developing solvent was a mixture of 0.3 *M* sodium chloride—acetone—10 *N* acetic acid (100:50:0.5, v/v). After development to about 10 cm, the plate was removed from the solvent tank, dried in a stream of hot air and sprayed with a mixture of 65% nitric acid and methanol (1:1, v/v [10]). The moist plate was then heated at 140°C for 10 min.

Densitometry

The location of the light-blue fluorescent spots, visible under long-wavelength (366 nm) ultraviolet light, were marked at the edge of the plate. The plates were scanned with a Shimadzu CS-910 TLC-scanner in a direction perpendicular to the direction of chromatography. Irradiation was at 360 nm with a Xenon lamp. The emitted light was quantitated after passing a 400-nm cut-off filter. The optical density was recorded and the peak areas were integrated with a Pye-Unicam DP 88 minigrator.

RESULTS

On the plate both compounds showed comparable fluorescent properties after the nitric acid treatment, i.e. an excitation optimum at about 360 nm. The fluorescence (as peak area) was linearly correlated with the concentration up to 200 ng, whereafter the calibration curve leveled off (Fig. 2). The lower limit of detection was at least 5 ng per spot. In the TLC system used the R_F values of TMP and TXP were identical (i.e. 0.61). For sulfamethoxazol and sulfadiazine R_F values of 0.37 and 0.55, respectively, were observed. Neither

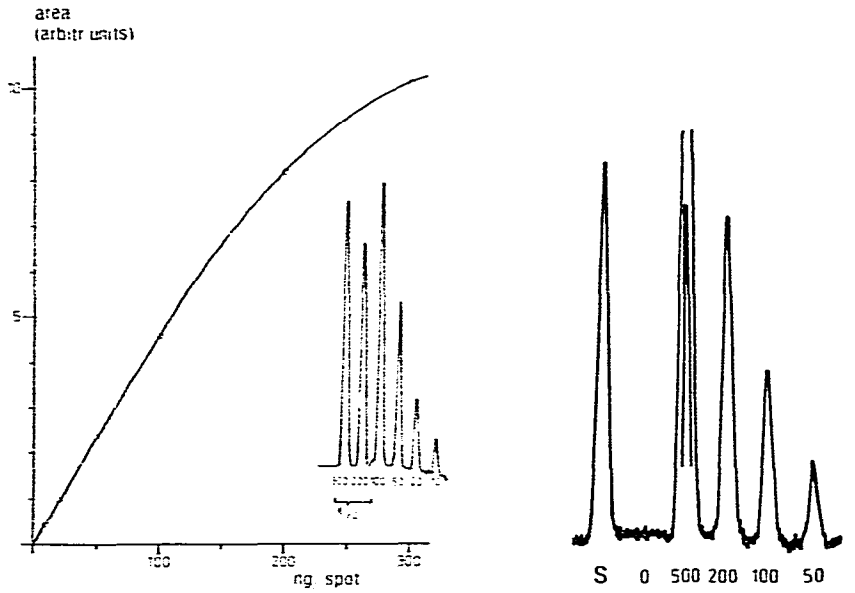

Fig. 2. Standard curve for TMP. Insert: recording of the fluorescence.

Fig. 3. Recording of the fluorescence of standard plasma TXP samples (ng/ml) and of a patient sample (S). The patient plasma sample was obtained from a female subject 15 h after the oral ingestion of 1 tablet of Tibirox^{R} (i.e. 100 mg of TXP and 250 mg of sulfadiazine). The TXP plasma concentration was found to be 270 ng/ml.

TABLE I

RECOVERY AND REPRODUCIBILITY OF TMP AND TXP FROM BIOLOGICAL SAMPLES

Compound	Concentration (μg/ml)	Sample	*n*	Recovery (%)	S.D. (%)
TMP	0.1	Plasma	4	84	8
	0.5	Plasma	6	83	5
	1.0	Plasma	4	82	3
TXP	0.1	Plasma	4	88	7
	0.3	Plasma	7	82	4.6
	1.0	Plasma	4	83	6
TMP	0.5	Urine	4	91	6
TXP	0.5	Urine	5	93	5
TMP*		Urine	5	102	8

*By direct application of 2 μl of undiluted urine on the plate. TMP concentration range 10—100 μg/ml.

the sulfonamides nor their N^4-acetyl derivatives nor any other constituent present in plasma extracts or urine developed fluorescent properties after the in situ nitric acid treatment. Hence the method allowed the estimation of plasma concentrations as low as 50 ng/ml easily (Fig. 3).

The method outlined proved to be fairly reliable; standard deviations for the reproducibility were less than 6%, the recovery from plasma for both the compounds was well beyond 80% (Table I).

DISCUSSION

Of the different chromatographic analytical methods, TLC has some merits that still make this technique an elegant analytical tool. For instance, the thin-layer chromatogram gives an overview of the total chromatogram, it can be stored for reinspection, several samples can be analysed on the same plate, etc. Concerning its quantitative power, especially in the case of fluorescent compounds, the sensitivity is in the nanogram range. This is shown in this paper, also, for the densitometric quantitation of TMP and TXP.

In comparison to the fluorometric TLC determination of TMP developed by Sigel and Grace [9], the present method has the advantage that analysis can be performed within a working day.

The nature of the fluorescent product or products resulting from the nitric acid treatment is not known. TLC of the fluorescent material gave no clear picture; mostly the chromatograms showed a trail of fluorescence. Treatment of TMP or TXP with hydrogen peroxide or nitrous acid (sodium nitrite and hydrogen chloride) did not lead to fluorescence. Haefelfinger [10] used a comparable nitric acid treatment to nitrate amitryptiline and nortriptyline on TLC. It may be that the fluorescent compound(s) are some nitrated products of TMP and TXP. On the other hand, pyrimethamine. another pyrimidine derivative with folic acid reductase inhibiting properties, did not develop fluorescence with this treatment (own observations).

The silanized silica gel plate as TLC system has the advantage that the eluting solvent is simple in composition (water, acetone mixtures) and its eluting power can be easily modulated by the addition of, for instance, electrolytes [11]. Also, aqueous samples can be easily applied. Resolution is mainly based on differences in partition [11].

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