Journal of Chromatography, 513 (1990) 392–396
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 515

Note

High-performance liquid chromatographic analysis of trimethoprim in the presence of its degradation products

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(First received September 25th, 1989; revised manuscript received April 23rd, 1990)

Various analytical methods have been used to determine trimethoprim in pharmaceuticals and biological fluids, including colorimetric analysis[1], first-derivative spectrophotometry[2], potentiometric analysis[3], polarography[4,5], gas–liquid chromatography[6,7], high-performance liquid chromatography (HPLC)[8–12] and thin-layer chromatography[13–17]. The last method[17] is claimed to be free from interferences by related and degradation products. Although with some of these procedures trimethoprim could be separated from its metabolites[5,10,13–15] with only one HPLC method trimethoprim was determined in the presence of two of its degradates[8].

We have succeeded in isolating five trimethoprim degradation products[18] and have published an HPLC analysis of trimethoprim in pharmaceuticals, using a Zorbax TMS column[19]. This method was free from interferences by the degradation products but when such a column was used during a kinetic study on samples containing trimethoprim in various buffer solutions, distortion of the trimethoprim peak occurred.

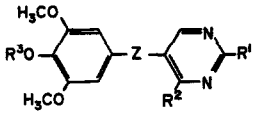
A trouble-free method, using a Partisil 10 ODS-3 column was then developed. The method was subsequently evaluated in the analyses of tablets and suspensions. This assay, which is described here, was found to be stability indicating and reliable.

EXPERIMENTAL

Degradation of trimethoprim

The degradation of trimethoprim and the isolation of degradation products (Fig. 1) were achieved as described previously[18].

0021-9673/90/$03.50



	R^1	R^2	R^3	Z
1	NH_2	NH_2	CH_3	$-CH_2-$
2	NH_2	NH_2	CH_3	$-\overset{O}{\overset{\|}{C}}-$
3	OH	OH	CH_3	$-CH_2-$
4	OH	NH_2	CH_3	$-CH_2-$
5	NH_2	OH	CH_3	$-CH_2-$
6	NH_2	OH	H	$-CH_2-$

Fig. 1. Structures of trimethoprim (1) and its degradation products.

Materials

Ammonium acetate (analytical grade) and acetonitrile (HPLC grade) both from Merck, South Africa, were used. Trimethoprim was supplied by Wellcome, South Africa.

Instrumentation

An M-45 dual-piston pump and a Model 441 fixed-wavelength detector at 254 nm, both from Waters Assoc. (Milford, MA, U.S.A.) were used. Sample injection was accomplished by means of a Rheodyne loop injector, Type 7012 equipped with a 20-μl loop. Peaks were integrated with a Waters 740 data module. Degassed mobile phase, consisting of acetonitrile (25%) and ammonium acetate (1%) in water at a flow-rate of 1 ml/min was used. A stainless-steel column (250 × 4.6 mm I.D.) packed with Partisil 10 ODS-3 (Whatman, Clifton, NJ, U.S.A.) was used.

Preparation of chromatographic solutions

Standard solutions containing 200–300 μg of trimethoprim per ml of methanol were prepared to obtain calibration graphs.

Sample solutions, to test the reproducibility of the method, were prepared using commercial tablets and a suspension (a single brand of each). Twenty tablets (100 mg of trimethoprim per tablet) were weighed, powdered and an amount of powder equivalent to 100 mg of trimethoprim was suspended in methanol, sonicated for 2 min, filtered and diluted with methanol to a concentration of about 250 μg of trimethoprim/ml. The suspension samples (50 mg of trimethoprim/5 ml) were diluted with methanol to the same concentration as above, sonicated and filtered.

Unfortified and fortified samples were prepared to evaluate the accuracy of the procedure. The unfortified tablet and suspension samples contained about 125 μg of trimethoprim/ml and were prepared in a similar manner as the sample solutions above.

Fortified sample solutions were prepared by spiking the unfortified samples with 60–120% of trimethoprim.

Analytical procedure

The analysis was performed in two separate stages. The first stage included all the tablet samples and the second stage the suspension samples. Six calibration samples (200–300 μg of trimethoprim/ml) were included in both the stages. All of the first stage solutions were chromatographed in sequence, followed by a second and third run after which the procedure was repeated with the suspension stage. The average of the three areas under the curves thus obtained was used in the calculations. *n*-Propyl *p*-hydroxybenzoate, present in the suspension and having a retention time of more than 50 min, can be eluted by flushing the column with acetonitrile for 6 min.

RESULTS AND DISCUSSION

A number of stationary phases were evaluated during the development of this method. Initially a Zorbax TMS column (DuPont, Wilmington, DE, U.S.A.) was used as it gave reproducible results with tablets and suspensions[19]. However, when this type of column was used during a kinetic study, distortion of the trimethoprim peak was evident in all samples containing buffer components (phosphates, sodium borate, potassium chloride, hydrochloric acid and sodium hydroxide). At lower buffer concentrations peaks tended to be flattened and broadened while higher buffer concentrations yielded completely distorted peaks. The reason for this distortion is not clear. It may possibly be attributed to the adsorption of inorganic ions to free silanol groups on the support which could then change the kinetics of molecular exchange. It was also found that the trimethoprim peaks obtained from suspension samples were flatter and slightly broader than those obtained from tablet samples. This phenomenon is probably due to various excipients in the suspension causing the same problem as mentioned above.

No peak distortion or change in theoretical plate count were observed when reverting back to trimethoprim solutions free of inorganic substances. These findings seem to indicate that peak distortion on the TMS column is the result of the composition of the sample rather than that of the mobile phase. Thus, although the mechanism which causes peak distortion is not known, it is clear that the process is reversible with no permanent damage to the column. These problems ruled out the TMS column for samples containing buffer components during a kinetic study.

A trouble-free method using a Partisil 10 ODS-3 column was subsequently developed. Baseline separation of trimethoprim in the presence of its degradates was achieved (Fig. 2a), which indicates that the method is stability-indicating. Identification of the five degradation products is possible in a single run. When this method was applied to various sample solutions during a kinetic study it was found that buffer components (hydrochloric acid, sodium hydroxide, sodium tetraborate, sodium acetate, citric acid, potassium dihydrogen phosphate, potassium chloride and trishydroxymethylaminomethane) caused no interference or peak distortion. The analysis was also carried out on trimethoprim–sulphamethoxazole combinations and it was found that sulphamethoxazole (retention time 3.9 min) presented no problem during the procedure. Chromatograms of the tablet and suspension samples are shown in Fig. 2b and c.

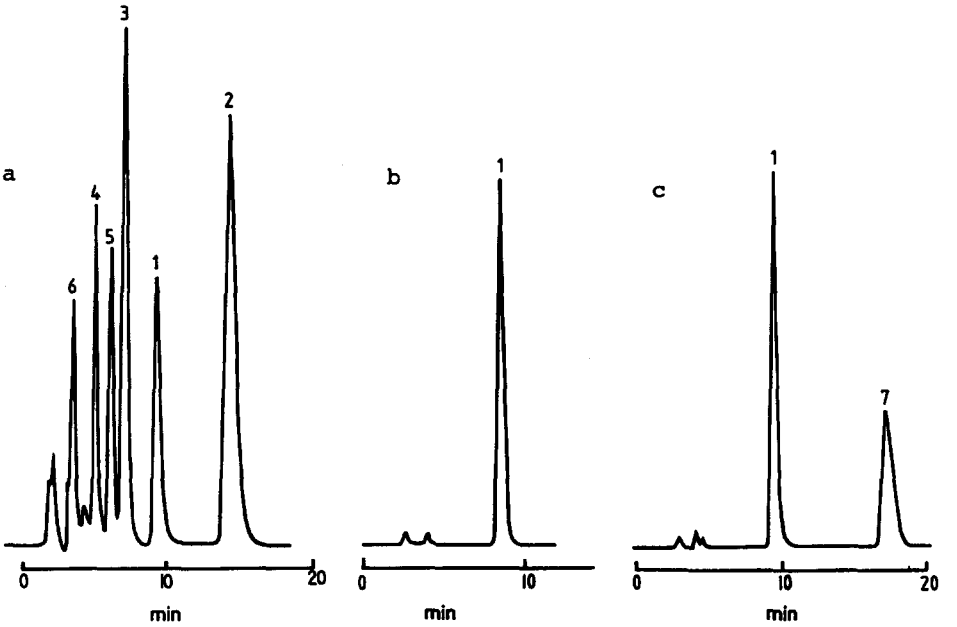


Fig. 2. (a) Chromatogram of trimethoprim (50 μg/ml) in the presence of its degradation products. Peak Nos. 1–6 refer to Fig. 1. (b and c) Chromatograms of trimethoprim in tablets (b) and suspension (c). Peak 7 = methyl *p*-hydroxybenzoate.

A Nucleosil C_{18} column (Machery-Nagel, Düren, F.R.G.) gave results similar to the Partisil ODS, the only exception being an incomplete separation between methyl *p*-hydroxybenzoate (present in the suspension) and compound 2 on the former column. This phenomenon however, did not influence the analysis of trimethoprim.

The pH of the mobile phase, which was adjusted to 6.90 ± 0.1 by ammonium acetate, is important for proper separation. While a pH value of 6.5 still rendered satisfactory results, baseline separation between trimethoprim and compound 5 (50 μg/ml of each) could not be achieved at pH values $\leqslant 6$.

Although a mobile phase containing 25% acetonitrile was used, the speed of analysis can be optimized by using the highest concentration of acetonitrile that will still effect baseline separation between trimethoprim and its degradates.

Calibration and calculation

A linear response was obtained for peak area *versus* concentration of the trimethoprim standard solutions between 200 and 300 μg/ml. The equations of the calibration graphs for six solutions were $y = 8.980x - 37.96$ ($r^2 = 0.9997$) and $y = 9.002x - 30.50$ ($r^2 = 0.9998$) for the tablet and suspension stages, respectively, where x is the concentration of trimethoprim and y the corresponding peak area.

The quantity of trimethoprim in the samples was calculated using the external standard method by substituting the peak area (y) in the appropriate equation above.

Range of linearity and detection limits

The detector response was found to be linear between 1.0–500 μg/ml (r^2 = 0.9999) while the limit of detection (signal-to-noise ratio 3:1) was 0.2 μg/ml.

Reproducibility

The coefficients of variation (C.V.) were 0.98 and 0.56%, respectively, for six tablet and six suspension samples. The average C.V. for three injections, calculated for all the chromatographed solutions, was 0.45%.

Accuracy

The average recovery, calculated from six fortified and six unfortified tablet samples was 99.4% (C.V. = 0.87%). The result for the same number of suspension samples was 99.1% (C.V. = 0.80%).

CONCLUSION

The method described for the quantification of trimethoprim in tablets and suspensions is simple, accurate and reproducible. Buffer components, sulphamethoxazole, methyl *p*-hydroxybenzoate and *n*-propyl *p*-hydroxybenzoate caused no interference during the analyses.

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