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Note

Comparison of colorimetric and high-performance liquid chromatographic determination of sulphamethoxazole and acetylsulphamethoxazole

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Sulphamethoxazole (SMX) is a sulphonamide derivative used in antibacterial chemotherapy alone or in combination with trimethoprim as co-trimoxazole. SMX, like other sulphonamides, is metabolized by acetylation, acetylsulphamethoxazole (Ac-SMX) being the major metabolite [1]. Methods using spectrophotometric [2], microbiological [3] and spectrofluorimetric [4] assays or pyrolysis gas chromatography–mass spectrometry [5] and high-performance liquid chromatography (HPLC) [6–8] have been described for the determination of SMX and Ac-SMX [9]. The colorimetric assay method of Bratton and Marshall [10] is usually used to determine sulphonamides and their metabolites. Since the conditions required for hydrolysis of acetylsulphonamides to sulphonamides in colorimetric assays can lead to incomplete hydrolysis and/or decomposition of sulphonamide originally present or formed during the experimental procedure, the use of HPLC for the determination of sulphonamide and its acetyl metabolite is now favoured [11–15].

The aims of the present study were to ascertain the effect of experimental conditions on Ac-SMX hydrolysis and to compare colorimetric and modified HPLC determinations of SMX and Ac-SMX.

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EXPERIMENTAL

Instrumentation

Chromatography was performed on a Waters (Model 6000 A) instrument equipped with an ultraviolet (UV) detector set at 254 nm and U6K loop injector. The μBondapak C_{18} column (5 μm particle size, 30 cm × 4.0 mm I.D.) was eluted with acetonitrile–acetic acid–water (19.5:1.0:79.5, v/v/v) at 1.7 ml/min. The mobile phase and the column were at room temperature (19–21°C). A Baush and Lomb spectrophotometer (Spectronic 20) was used for the colorimetric assay.

Chemicals and reagents

HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.) and all other chemicals were analytical grade. SMX and Ac-SMX were gifts from Roche (Istanbul, Turkey). Sulphadimidine (SDD) was purchased from Sigma (St. Louis, MO, U.S.A.).

Sample collection

SMX (10 mg/kg) was administered orally to three subjects after an overnight fast. A 10-ml blood sample was withdrawn into Vacutainer tubes prior to and 3, 5 and 7 h after taking the drug. The tubes were centrifuged at 1500 *g* for 10 min and the serum was removed. Serum samples were stored at −20°C for subsequent analysis.

Chromatographic assay

Serum (0.2 ml) diluted 1:2 (v/v) with distilled water. Then 0.4 ml of trichloroacetic acid (TCA) (5%, w/v) was added. The solution was thoroughly mixed on a vortex-mixer. After centrifugation at 4000 *g* for 5 min, 10 μl of supernatant were injected into the column. SDD (12.5 μg/ml) was used as internal standard. Calibration curves were prepared by plotting peak-area ratios for SMX/SDD and Ac-SMX/SDD against the known concentrations of the sulphonamides.

Colorimetric assay

SMX and Ac-SMX were determined in duplicate by the Bratton–Marshall procedure [10]. The procedure for the determination of SMX in standard solutions and human serum samples was as follows. Add 0.2 ml of serum to 2.0 ml of 10% (w/v) TCA, vortex-mix and centrifuge for 5 min at 4000 *g*. Transfer a 1.0-ml aliquot of supernatant to a glass-stoppered tube and add 0.05 ml of 4 *M* hydrochloric acid. Heat the tube at 100°C for 60 min, then cool it in cold water and allow it to come to room temperature (19–21°C). Then add 0.1 ml of sodium nitrite solution (0.1%, w/v) to both heated and non-heated tubes, 3 min later add 0.1 ml of ammonium sulphamate (0.5%, w/v), and a further 2

min later add 0.1 ml of N-(1-naphthyl)ethylenediamine dihydrochloride solution(1%, w/v). Then allow the tube to stand for colour development for at least 15 min in the dark before measuring the absorbance at 540 nm. The Ac-SMX concentrations of standard solutions and samples were calculated as the difference between the absorbance values for heated and non-heated tubes.

Linearity

The stock solutions of SMX and Ac-SMX were diluted to 3.125, 6.25, 12.5, 25, 50, 100 and 200 μg/ml with drug-free human serum. Standards containing these concentrations were assayed by colorimetry or HPLC. The standard curves were analysed by linear regression analysis to determine linearity. SMX standards were stable at −70 °C for at least 4 months and Ac-SMX standards were stable 5 °C for at least 1 month.

Precision

The precision of the colorimetric and HPLC methods were obtained by determination of six aliquots of SMX and Ac-SMX in drug-free human serum. One concentration for both SMX and Ac-SMX was used, and six aliquots at each concentration were assayed in one batch (intra-assay precision).

Accuracy

Known amounts of SMX and Ac-SMX were dissolved in drug-free human serum and assayed six times in an encoded fashion.

Recovery

Identical amounts of SMX and Ac-SMX were dissolved in distilled water or drug-free human serum and assayed. In the chromatographic method, drug peak areas in drug-free human serum were compared with drug areas in distilled water.

Ac-SMX hydrolysis

A 100 μg/ml solution of Ac-SMX was prepared in drug-free human serum, then the following procedure was performed. Ac-SMX solution (0.2 ml) was added to 2.0 ml of 10% (w/v) TCA solution (A) or to 2.0 ml distilled water (B) and vortex-mixed. Aliquots (1.0 ml each) were transferred to glass-stoppered tubes, and 0.05 ml of 4 *M* hydrochloric acid (C) or 0.05 ml of distilled water (D) was added. Tubes (A+C), (A+D) and (B+C) were considered as TCA+hydrochloric acid, TCA and hydrochloric acid, respectively. Then tubes were heated at 100 °C for 15 min, 30 min and 60 min, cooled in cold water and allowed to come to room temperature (19–21 °C). Aliquots (10 μl) from each tube injected into the HPLC column. The calibration curves prepared by plotting peak areas of SMX and Ac-SMX against known concentrations were used for quantitation.

RESULTS AND DISCUSSION

Comparison of the HPLC and colorimetric analyses

HPLC results. Extraction of drug-free human serum indicated that there was no interference and all peaks were resolved, as illustrated in Fig. 1. The calibration curves of SMX ($y=0.0756+0.0404x$) and Ac-SMX ($y=-0.0087+0.0442x$) were linear over the range 3.125–200 μg/ml for Ac-SMX. The mean correlation coefficients for SMX and Ac-SMX obtained from six analytical runs were 0.9964 and 0.9976, respectively. The inter-assay precision, accuracy and recovery of SMX and Ac-SMX measurements at 40 μg/ml were calculated and are presented in Table I. These data indicate that the chromatographic assay procedure for SMX and Ac-SMX in serum was reliable, accurate and reproducible. Results also revealed that plasma concentrations of SMX and Ac-SMX achieved after oral administration of SMX can easily be determined by the present modified HPLC method. The mean minimal steady-state plasma concentrations of SMX and its acetyl metabolite were

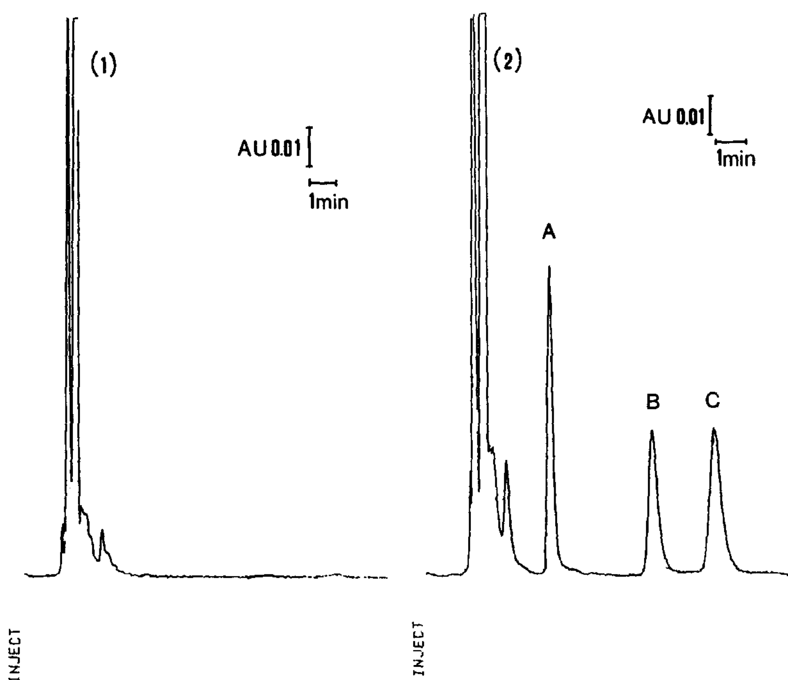

Fig. 1. Chromatograms of drug-free human serum and standard serum samples: (1) drug-free human serum extract; (2) standard serum containing 12.5 μg/ml sulphadimidine (SDD), 25 μg/ml sulphamethoxazole (SMX) and 25 μg/ml acetylsulphamethoxazole (Ac-SMX). Peaks: A = SDD (4.4 min); B = SMX (7.2 min); C = Ac-SMX (9.3 min).

TABLE I

PRECISION, ACCURACY AND RECOVERY OF SMX AND Ac-SMX BY HPLC

In all cases, $n=6$.

Compound	Precision (%)	Accuracy (%)	Recovery (%)
SMX	2.6	98.4	97.3
Ac-SMX	3.6	93.1	91.3

TABLE II

PRECISION AND ACCURACY OF THE QUANTITATION OF Ac-SMX AND SMX MIXTURES BY COLORIMETRY

In all cases, $n=6$.

Concentration (μg/ml)					S.D.	Variance	C.V. (%)
Added			Obtained[a]				
SMX	Ac-SMX		SMX	Ac-SMX			
45	5	Unheated	40.6	–	3.2	10.2	7.9
		Heated	47.7	–	6.8	46.2	18.4
		Difference	–	7.1	7.5	56.4	105.8
37.5	12.5	Unheated	33.6	–	3.1	9.6	8.8
		Heated	46.2	–	1.4	2.0	3.0
		Difference	–	12.6	3.4	11.6	27.0
12.5	37.5	Unheated	11.6	–	1.3	1.7	11.2
		Heated	44.7	–	4.6	21.2	10.3
		Difference	–	33.1	4.8	22.9	14.5
5	45	Unheated	4.6	–	0.6	0.4	13.0
		Heated	44.8	–	2.4	5.8	5.4
		Difference	–	40.2	2.5	6.2	6.2

[a]Mean of six determinations at each concentration.

reported to be 11.0 ± 5.4 μg/ml and 34.9 ± 3.2 μg/ml, respectively [16], within the concentration range studied in the present study.

Colorimetric results. The calibration curve of SMX ($y=0.0036+0.0141x$; $r=0.9997$) was linear over the range 3.125–200 μg/ml. The concentration of Ac-SMX was calculated by using the SMX calibration curve after the hydrolysis procedure. The precision and accuracy of SMX and Ac-SMX measurements at different concentrations were calculated and are shown in Table II. The principal disadvantage of the colorimetric method is the measurement of

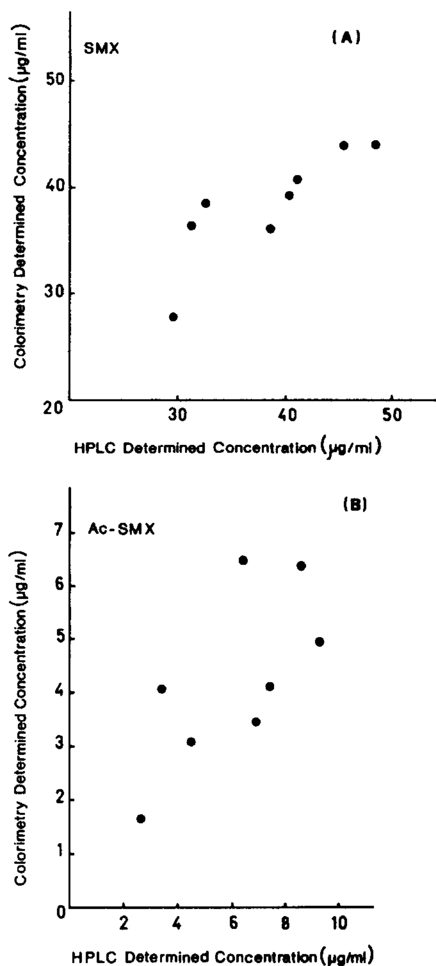

Fig. 2. Comparisons of serum SMX (A) and serum Ac-SMX (B) concentrations determined by both colorimetry and HPLC after oral administration of sulphamethoxazole (10 mg/kg) to three human subjects. SMX: $y = 1.258 + 0.967x$; $r = 0.7974$. Ac-SMX: $y = 1.876 + 0.976x$; $r = 0.6897$.

metabolite concentration by the difference between heated and non-heated samples. The variance of metabolite concentrations was calculated from the sum of variances of both measurements. The coefficients of variation (C.V.) of values obtained by difference measurements are high, especially at low metabolite concentrations.

Serum samples obtained from healthy subjects given SMX were analysed for their drug and metabolite content using both HPLC and colorimetry. Correlation analysis yielded correlation coefficients of 0.7974 and 0.6897 for SMX and Ac-SMX, respectively (Fig. 2).

Effect of heat and acid on Ac-SMX hydrolysis

The heat and acidic strength of the medium are crucial parameters for the Bratton–Marshall colorimetric assay, which is based on hydrolysis of acetylsulphonamide to sulphonamide. It is suggested that the sulphonamide nucleus can decompose when the originally described conditions are used, and that this may lead to inaccurate determination of sulphonamide and/or its acetylated metabolite [7]. Incubation of samples for a shorter time and/or in a less acidic medium may reduce the possible loss of these substances. However, Whelpton et al. [17] reported that incomplete hydrolysis of acetylsulphadimidine (Ac-SDD) occurred when the samples were heated at 100°C for a shorter duration (15 min). A series of experiments was performed to show the effects of both heat and acid on hydrolysis of Ac-SMX. A 100 μg/ml solution of Ac-SMX was incubated for different periods of time, and samples were taken and analysed by HPLC. The time-course of the reaction revealed that a shorter incubation period and lower acidic strength resulted in incomplete hydrolysis of Ac-SMX. For example, 38.0% of Ac-SMX did not hydrolyse to SMX after incubation for 15 min in TCA. Furthermore, even after incubation of Ac-SMX in the routinely used hydrolysis condition (60 min, in TCA+HCl) ca. 10% of Ac-SMX was not hydrolysed to SMX (Fig. 3).

Another series of experiments was done to assess the undesirable decom-

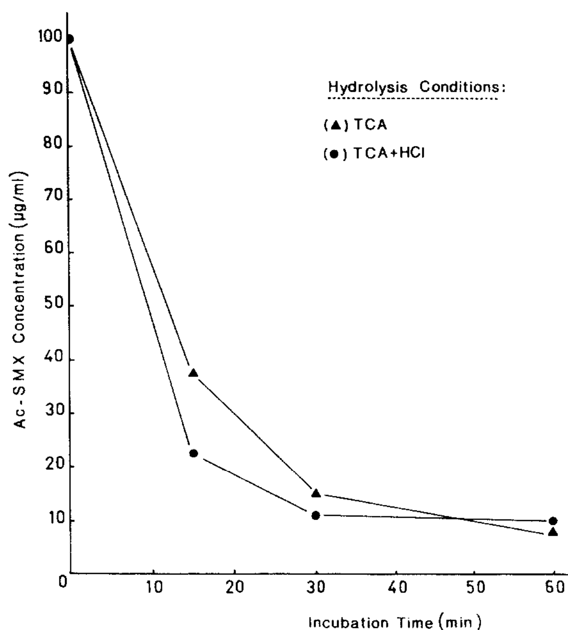

Fig. 3. Effects of acidity and incubation time on the hydrolysis of Ac-SMX (100 μg/ml). Determinations were performed by HPLC.

position of SMX. Aliquots were taken from Bratton–Marshall hydrolysis media, before and after incubation of SMX solutions, and analysed by HPLC. The mean recoveries of SMX at concentrations ranging from 12.5 to 100 μg/ml were 101.3 ± 4.2% (4.2% C.V.), and no extra peak was observed in the chromatograms. This suggested that there was no decomposition of SMX in the hydrolysis medium at this concentration range.

CONCLUSION

The HPLC method is more reliable and accurate than the conventional Bratton–Marshall colorimetric method for determination of SMX and its acetyl metabolite. The major disadvantage of the colorimetric method is the indirect measurement of the Ac-SMX after hydrolysis. In addition, Ac-SMX hydrolyses incompletely under the hydrolysis conditions routinely used in the colorimetric assay.

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