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SIMULTANEOUS DETERMINATION OF AZATHIOPRINE AND 6-MERCAPTOPURINE IN SERUM BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The simultaneous determination of azathioprine and its metabolite 6-mercaptopurine in serum by reversed-phase high-performance liquid chromatography is described. 6-Mercaptopurine was converted to a derivative, 6-mercaptopurine-N-ethylmaleimide, which is stable against autoxidation, on reaction with N-ethylmaleimide. Since the N-ethylmaleimide derivative was more hydrophobic than the parent compound, it could be extracted into ethyl acetate together with azathioprine and the derivative was retained on the reversed-phase column better than 6-mercaptopurine. In addition, 6-mercaptopurine-N-ethylmaleimide absorbed at the same wavelength (280 nm) as azathioprine. Consequently, this derivatization procedure enabled the simultaneous extraction, separation, and detection of these compounds.

INTRODUCTION

Azathioprine [AZA, 6-(1-methyl-4-nitroimidazol-5-ylthio)-purine] has been utilized as an effective immunosuppressive agent in organ transplantation and it is transformed in vivo to the active moiety, 6-mercaptopurine (6MP). 6MP itself is also used as an antiplastic agent. Their use should be carefully controlled in order to avoid severe adverse effects. In addition, there are great interindividual differences in the sensitivity and tolerance to these drugs. These facts prompted us to investigate the simultaneous monitoring of AZA and 6MP.

Numerous methods for the determination of AZA and/or 6MP have been proposed such as colorimetry and ultraviolet (UV) spectrometry involving ion-exchange column chromatography [1—3], but these methods gave only poor sensitivity. Although gas chromatography—mass spectrometry [4, 5] was reported as a sensitive tool for the assay of 6 MP, the need to use a mass spectrometer has prevented its wide application in routine laboratories. High-

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performance liquid chromatography (HPLC) on ion-exchange column [6—8] has been limited to the analysis of 6MP and it has not been applied to routine drug monitoring. Reversed-phase HPLC [9—11] has recently been introduced in this field to give high sensitivity and selectivity, but the simultaneous determination of AZA and 6MP has not yet been achieved by this method. This may be due to the fact that their absorption maxima are a long way apart, that their retention times differ greatly from each other, and that the mercapto group of 6MP is highly sensitive to autoxidation and it should be treated in the presence of a stabilizing agent which also causes the decomposition of AZA. In addition, 6MP is hardly soluble in organic solvents and it cannot be extracted together with AZA. Perchloric acid or trichloroacetic acid are effective for the extraction of 6MP, but the use of strong acid necessitated a tedious neutralization step in the course of sample pretreatment.

In the present study, 6MP was converted to a stable derivative (6MP-NEM) by reaction with N-ethylmaleimide (NEM) prior to chromatography. This technique made the addition of the stabilizing agent unnecessary, and solved the problems caused by the differences in absorption wavelengths, retention times, and solubilities between the two compounds. As a result, the simultaneous separation and determination of AZA and 6MP on reversed-phase HPLC was achieved.

EXPERIMENTAL

Materials

AZA was obtained from Nippon Wellcome (Osaka, Japan) and 6MP was purchased from Sigma (St. Louis, MO, U.S.A.). 2-Ethyl-4-oxoquinazoline (2EOQ) was the gift of Dr. Y. Okamoto in our laboratory. Stock solutions of these compounds were prepared by dissolving 5 mg in 50 ml of ethanol and storing at 4°C. The NEM reagent was prepared fresh daily by dissolving 30 mg of NEM (Wako, Osaka, Japan) in 2 ml of 0.05 *M* phosphate buffer, pH 7.0. HPLC-grade acetonitrile was obtained from Kanto (Tokyo, Japan). Mobile phase A was 0.01 *M* KH_2PO_4 aqueous solution containing 9% acetonitrile; mobile phase B was 0.01 *M* KH_2PO_4 aqueous solution containing 50% acetonitrile. Other reagents and solvents were of analytical grade. Hyland Q-Pak Chemistry Control Serum I was purchased from Travenol Labs. (IL, U.S.A.).

Procedure

To 200 μl of serum were added 5 μl of ethanol containing 250 ng of 2EOQ as the internal standard. The sample was mixed with 100 μl of the NEM reagent solution and allowed to stand at room temperature for 1 h. Then 1.8 ml of ethyl acetate were added, and the solution was mixed by an automatic mixer. The resulting mixture was then centrifuged at 1800 *g* for 5 min. A 1.5-ml aliquot of the supernatant was transferred to another centrifuge tube. After duplicate extractions, the organic phases were combined and evaporated to dryness under reduced pressure below 30°C. The residue was dissolved in 100 μl of mobile phase A and a 90-μl aliquot of the solution was injected into the chromatograph.

Chromatographic procedure

The liquid chromatograph consisted of a Waters Model 6000A pump, a U6K universal injector, a μBondapak C_{18} (particle size, 10 μm) column (all from Waters Assoc., Milford, MA, U.S.A.) equipped with a Soma UV detector S-310 A (Soma Optic Co., Tokyo, Japan) operated at 280 nm, and a Shimadzu Model R-111 recorder (Shimadzu, Kyoto, Japan) with a range of 1 mV under the full-scale setting of 0.005 or 0.002. The column was first eluted with mobile phase A for 26 min to separate AZA and 6MP-NEM and then eluted with mobile phase B for 1 min to wash the column. Both mobile phases were delivered at a constant flow-rate of 1.5 ml/min.

Extraction recoveries

The extraction recoveries of AZA and 6MP were examined using sera spiked with 500 ng/ml and 100 ng/ml of these compounds according to the procedure. The peak heights for spiked AZA were compared with the peak heights obtained from the same amount of standard. Also, the peak heights for 6MP-NEM were compared to the peak heights obtained from the same amount of 6MP that had been derivatized but not extracted.

Identification of the NEM derivative of 6MP

After 6MP was allowed to react with the NEM reagent according to the procedure, 6MP-NEM was purified by injecting the reaction mixture into the chromatograph and eluting with mobile phase A. The fraction of 6MP-NEM was collected and extracted with ethyl acetate. The extract was evaporated to dryness. The product was identified as 6MP-NEM by mass spectrometry. 6MP-NEM: *m/z* (percentage relative abundance) = 110 (76); 125 (44); 152 (100); 277 (M^+, 8).

RESULTS AND DISCUSSION

Absorption spectra of AZA, 6MP and 6MP-NEM in mobile phase A are shown in Fig. 1. Although the absorption maximum of 6MP is located away from that of AZA, that of 6MP-NEM appears at the same wavelength (280 nm) as that of AZA.

The reaction conditions for the pre-column derivatization of 6MP with NEM were examined using 500 ng/ml 6MP at room temperature. AZA remained unchanged during the derivatization. Fig. 2 shows the peak height of the 6MP derivative plotted against the concentration of NEM in the derivatization reagent. The peak height increased with the NEM concentration and continued to increase above 15 mg/ml NEM, but 15 mg/ml NEM was adopted in the assay procedure because the solubility of NEM is limited. The peak height also increased with the reaction time until 120 min, but 60 min was adopted in the procedure to minimize the time for the pretreatment.

AZA can be extracted with ethyl acetate or acetonitrile but 6MP cannot be extracted with such organic solvents. Therefore, perchloric acid and trichloroacetic acid have been used for the pretreatment of biological samples. However, the use of these strong acids was always followed by tedious neutralization steps. On the other hand, 6MP-NEM could be readily extracted with

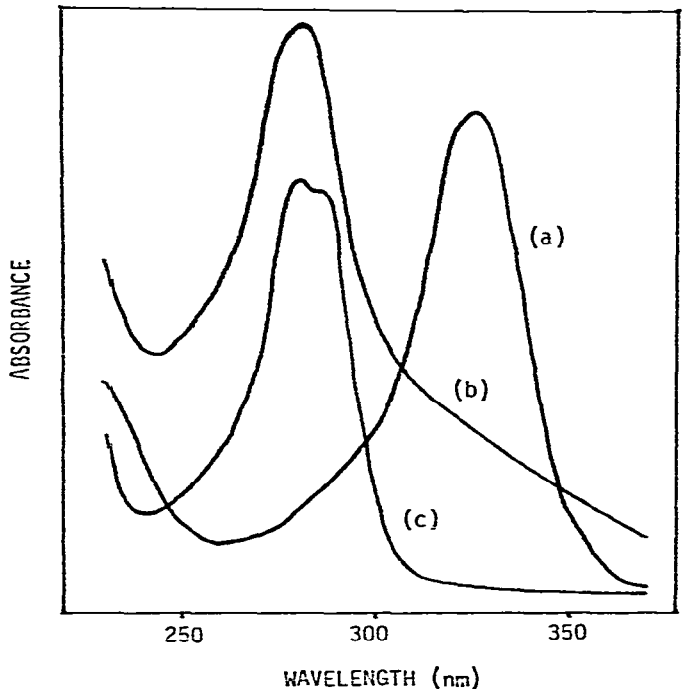

Fig. 1. Absorption spectra of (a) 6MP, (b) AZA and (c) 6MP-NEM dissolved in mobile phase A.

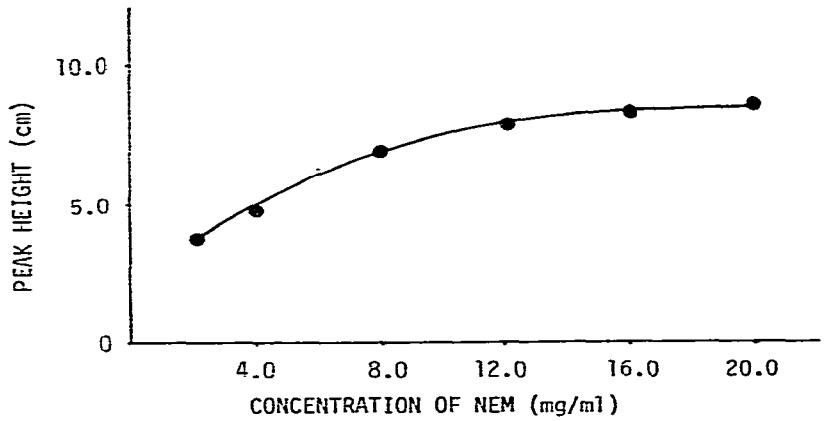

Fig. 2. The peak height of 6MP-NEM plotted against the concentration of NEM in the derivatization reagent.

ethyl acetate. The extraction of AZA and 6MP was examined using volumes of ethyl acetate ranging from 0.6 ml to 2.4 ml. Satisfactory recovery was obtained using 1.8 ml of ethyl acetate as listed in Table I.

Fig. 3a shows the chromatogram obtained from a standard mixture of AZA, 6MP and the internal standard (2EOQ) without extraction. The retention times of AZA, 6MP-NEM and 2EOQ were 13.4, 18.6 and 28 min, respectively. Fig. 3b and c display chromatograms of the ethyl acetate extracts of serum

TABLE I

EXTRACTION RECOVERIES OF AZA AND 6MP FROM SERUM

Values (in per cent) are expressed as mean ± S.D., $n = 5$.

Compound	Concentration	
	500 ng/ml	100 ng/ml
AZA	98.3 ± 3.72	87.2 ± 7.73
6MP	91.8 ± 4.78	82.9 ± 6.91

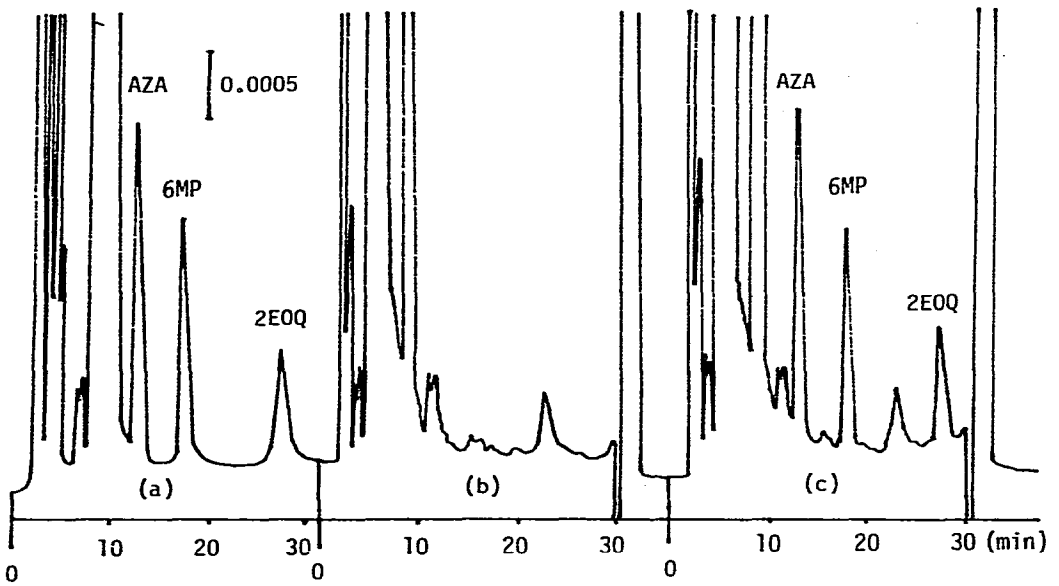

Fig. 3. Chromatograms obtained from: (a) a standard mixture of AZA (50 ng), 6MP (50 ng) and internal standard (250 ng) without extraction; (b) serum blank; and (c) serum spiked with AZA (50 ng) and 6MP (50 ng) with extraction according to the procedure.

blank and serum spiked with AZA, 6MP and 2EOQ, respectively. Since the serum samples show big unknown peaks at a retention time of about 45 min when eluted with mobile phase A, the mobile phase was changed to B at 26 min to reduce the separation time. This procedure enabled the injection of the next sample 40 min after the previous injection. AZA, 6MP-NEM and the internal standard were separated well from serum components, whereas the peak of underivatized 6MP appeared at the retention time of 3 min and overlapped with those of serum components. This indicates that the derivatization of 6MP is also useful for the removal of the peak of 6MP from the region where interfering peaks of serum components appear.

Standard curves for AZA and 6MP were linear in the range 10—500 ng/ml and passed through the origin. The signal-to-noise ratios of AZA and 6MP at a concentration of 10 ng/ml were 5.8 and 2.5 with 0.002 a.u.f.s., respectively. In addition, 2 ng/sample each of AZA and 6MP in serum was found to be detectable as shown in Fig. 4.

The within-run and day-to-day variability of the assay for AZA and 6MP

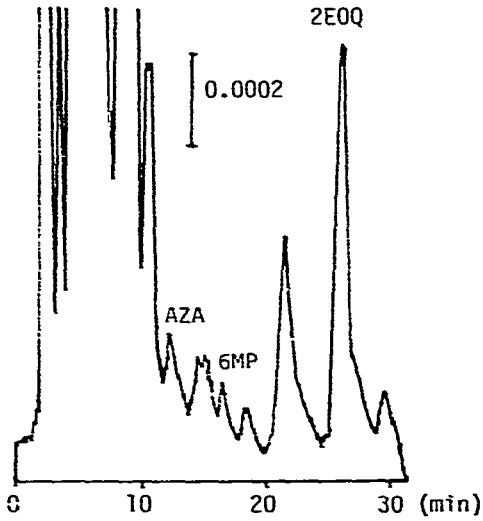

Fig. 4. Chromatogram obtained from serum spiked with AZA (2 ng) and 6MP (2 ng) with extraction according to the procedure.

TABLE II

COEFFICIENTS OF VARIATION (%) FOR ANALYSES

$n = 5$.

Compound	Concentration (ng/ml)	C.V. (%)	
		Within-run	Day-to-day
AZA	250	1.96	2.65
	50	2.64	4.92
6MP	250	3.39	4.98
	50	4.25	5.78

were studied as summarized in Table II. The variabilities resulted in coefficients of variation of less than 6%.

CONCLUSION

The present method involving the derivatization of 6MP offers the following advantages: (1) derivatized 6MP can be extracted together with AZA into ethyl acetate giving excellent recovery; (2) the absorption maximum of 6MP appears at 280 nm at which wavelength AZA also absorbs; (3) 6MP-NEM is well retained on the reversed-phase column and appears close to AZA on the chromatogram; (4) the stabilizing agent needed for the mercapto group can be eliminated.

The present method thus facilitates the rapid and sensitive simultaneous determination of AZA and 6MP.

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REFERENCES

1 T.L. Loo, J.K. Luce, M.P. Sullivan and E. Frei, III, Clin. Pharmacol. Ther., 9 (1967) 180.
2 J.J. Coffey, C.A. White, A.B. Lesk, W.I. Rogers and A.A. Serpick, Cancer Res., 32 (1972) 1283.
3 A.H. Chalmers, Biochem. Med., 12 (1975) 234.
4 J.M. Rosenfeld, V.Y. Taguchi, B.L. Hillcoat and M. Kawai, Anal. Chem., 49 (1977) 725.
5 S. Floberg, P. Hartvig, B. Lindström, G. Lönnerholm and B. Odlind, J. Chromatogr., 225 (1981) 73.
6 D.J. Nelson, C.J.L. Bugge, H.C. Krasny and T.P. Zimmerman, J. Chromatogr., 77 (1973) 181.
7 H.J. Breter and R.K. Zahn, J. Chromatogr., 137 (1977) 61.
8 H.J. Breter, Anal. Biochem., 80 (1977) 9.
9 J.L. Day, L. Tterlikkis, R. Niemann, A. Mobley and C. Spikes, J. Pharm. Sci., 67 (1978) 1027.
10 T.L. Ding and L.Z. Benet, J. Chromatogr., 163 (1979) 281.
11 S.N. Lin, K. Jessup, M. Floyd, T.P.F. Wang, C.T. van Buren, R.M. Caprioli and B.D. Kahan, Transplantation, 29 (1980) 290.