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Note

High-performance liquid chromatographic determination of amphotericin B in human urine

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Amphotericin B (AMB), an antifungal antibiotic with a polyene structure [1], is often used to treat systemic fungal infections [2]. A recent paper reported the increased incidence of urinary fungaria in diabetic patients, varying with the duration and severity of the disease (diabetes mellitus) and the patient's age [3]. AMB can be useful in treating such infection, but a large amount (70–80%) of orally administered AMB is not absorbed by the gut [4], resulting in a low blood level [5] and poor excretion in urine [4]. Therefore, for effective treatment of urinary fungaria and the prevention of significant side-effects, such as nephrotoxicity, AMB levels in the blood as well as the urine need to be monitored [6]. Unfortunately, little information is presently available on AMB excretion and the dynamics of urine AMB levels after oral administration of the drug.

Recently, rapid, accurate and reproducible high-performance liquid chromatographic (HPLC) methods have been developed for the determination of blood AMB levels [7–11]. However, it has been difficult to establish a precise assay method for the urine AMB concentration because the level itself is low (ng/ml) and because there are other yellow components of high colour intensity in the urine that display similar solubility to AMB and sensitivity to light, heat and air [12]. The assay of urine AMB has been tried by a microbiological method [13–15], but the level is too low to allow monitoring of a patient who has received the drug orally. Moreover, the bioassay is not very precise [14] and is difficult to interpret, especially at such low drug levels.

In the present study, we developed a sensitive and precise HPLC method for determining the urine AMB level after removing the colour components in the urine and concentrating the AMB. This method makes it possible, for the first

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time, to monitor precisely the urine AMB level in patients who receive the drug orally. We also used the present method to study the dynamics of urine AMB levels in six diabetic patients with urinary fungal infections.

EXPERIMENTAL

Apparatus

A high-performance liquid chromatograph (Trirotar, Japan Spectroscopic, Tokyo, Japan) was used with a reversed-phase μBondapak C_{18} (10 μm average particle size, Waters Assoc., Milford, MA, U.S.A.) stainless-steel column (30×0.4 cm I.D.). The detector was a variable-wavelength spectrophotometric detector (UVIDEC 100-VI, Japan Spectroscopic). The absorption spectrum of AMB was measured with a spectrophotometer (Hitachi 220, Hitachi, Tokyo, Japan). The urine sample was concentrated using a cartridge packed with reversed-phase packing (Sep-Pak C_{18}, Waters Assoc.).

Chemicals and standards

A pure reference sample of AMB was purchased from E.R. Squibb & Sons (Princeton, NJ, U.S.A.). Acetonitrile, tetrahydrofuran, dimethyl sulphoxide, methanol and all other chemicals were of analytical-reagent grade from Wako Pure Chemicals (Osaka, Japan). A stock standard solution of AMB (500 μg/ml) was prepared in dimethyl sulphoxide, and a working standard solution (200 μg/ml) was prepared by dilution of the stock solution with methanol. Control samples were prepared from drug-free pooled urine to give AMB concentrations of 0.5–10 ng/ml.

Preparation of HPLC sample

A urine sample (25 or 50 ml) was centrifuged at 1400 *g* for 10 min, then passed through a Sep-Pak C_{18} cartridge. The cartridge was then washed with 20 ml of 50% aqueous methanol. AMB was eluted with 2.5 ml of absolute methanol, and the eluent was evaporated to dryness under a stream of nitrogen at 65°C. The residue was redissolved in 0.5 ml of methanol. An 80-μl portion of the methanol solution was injected into the chromatograph. A methanol solution containing 200 ng/ml AMB was prepared as a control for determining the AMB concentration from the peak height.

The HPLC conditions were as follows: column, μBondapak C_{18} (10 μm); mobile phase, 10 m*M* acetate buffer (pH 7.0)–acetonitrile–methanol–tetrahydrofuran (50:25:20:5, v/v); detection wavelength, 405 nm; flow-rate, 1.5 ml/min; column temperature, ambient (25–27°C); a.u.f.s., 0.0025; sample volume, 80 μl. Typical chromatograms of a blank urine and a urine sample containing 4 ng/ml AMB are shown in Fig. 1a and b.

Urinary excretion of AMB in patients

A suspension of AMB (Fungizone®, 4800 mg of AMB) was administered orally before breakfast to six diabetic patients (age, 21–79 years; body weight, 55.8–72.0 kg; fasting blood glucose, 62–275 mg per 100 ml) with urinary fungal infections. The drug was administered every morning for five days. Urine samples were pooled

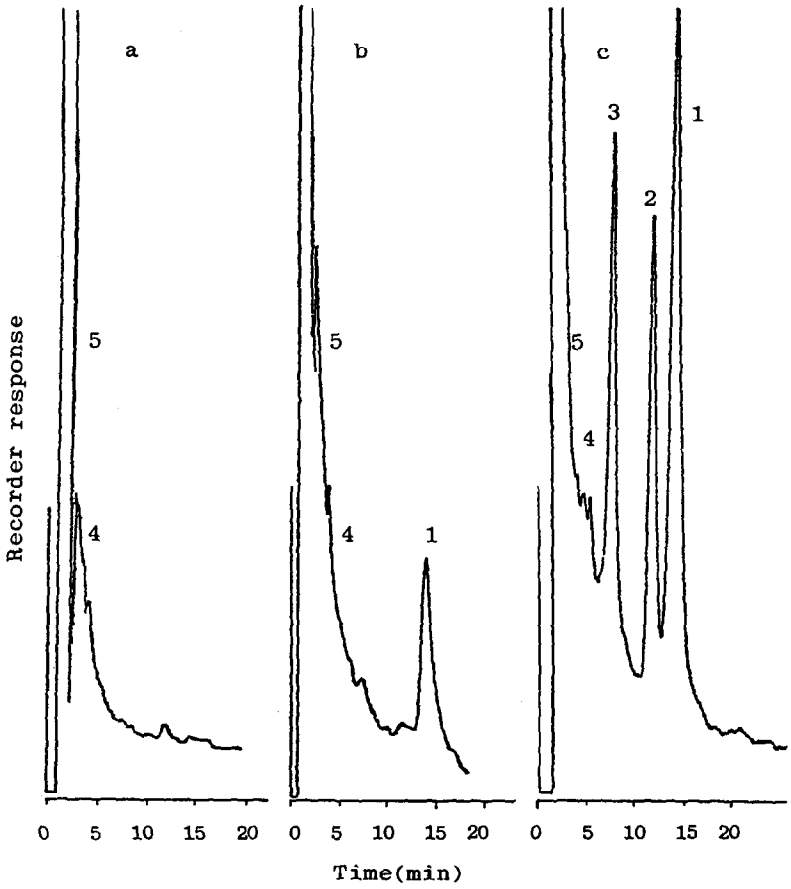

Fig. 1. HPLC profiles of blank urine and urine sample containing AMB. (a) Blank urine; (b) AMB added to urine (4 ng/ml); (c) urine sample from patient to whom AMB was administered. Peaks: 1 = AMB; 2 and 3 = unknown compounds; 4 and 5 = urine colour components. For b urine was prepared by diluting the stock standard solution with pooled urine.

over 24 h after the drug administration, and all samples were stored at −20°C until assay.

Calibration curve

Standard samples (0.5–8.0 ng/ml) for the calibration curve were prepared by diluting the stock solution (500 μg/ml) with pooled human urine that did not contain the drug. The chromatographic peak height of AMB was subjected to linear regression versus the corresponding AMB concentrations.

RESULTS AND DISCUSSION

To determine urine AMB levels, we needed to overcome two problems. First, the urine AMB level is lower than that in serum, and thus a more sensitive method had to be developed. Second, a large amount of colour components with a colour

TABLE I

EFFECT OF WASHING WITH METHANOL ON AMB RECOVERY

A sample solution (25 ml, 10 ng AMB per ml pooled urine) was centrifuged at 1400 *g* for 10 min, then passed through a Sep-Pak C_{18} cartridge washed with 20 ml of aqueous methanol (0–70%). Next, the AMB was eluted with 2.5 ml of absolute methanol, and its concentration in the eluate was determined by HPLC.

Methanol concentration (%)	AMB concentration (ng/ml)		Recovery (%)
	Calculated	Found	
0	10	8.6	86*
25	10	9.4	94
40	10	9.7	97
50	10	10.0	100
60	10	9.5	95
70	10	4.3	43

*Chromatogram showed interference from a large amount of colour components in the urine.

intensity similar to that of AMB is present in the urine sample, and this amount must be reduced before HPLC analysis. In the present study, a cartridge packed with a reversed-phase packing (Sep-Pak C_{18}) was successfully used to remove the colour components and to raise the AMB concentration 50- to 100-fold. Cartridges on which AMB and the colour components were absorbed were washed with aqueous solutions containing various concentrations of methanol (Table I), followed by elution of AMB with methanol. An aqueous solution containing 70% methanol effectively eliminated the colour components, but the recovery of AMB was low (43%). When 50% methanol was used, the colour components were not completely eliminated, but their level was reduced, and optimal recovery (100%) of AMB was attained.

Table II shows the effects of streams of air and nitrogen on the recovery of AMB when the eluent from the cartridge was evaporated to dryness. Under an air stream at 65°C, the average recovery of AMB was low (73%) with large variation (coefficient of variation, C.V., 27.6%). On the other hand, the nitrogen stream led to a high recovery of AMB (99.4%) with a small C.V. value (1.1%), suggesting that AMB is sensitive to air.

When the mobile phase (acetonitrile–10 m*M* acetate buffer pH 7.0, 39:61, v/v) described in our previous paper [11] on HPLC analysis of serum AMB was applied to the present HPLC method, AMB gave a retention time of 13 min; however, urine colour components were continuously eluted up to 30 min, overlapping with the AMB peak. As shown in Table III, addition of methanol remarkably retarded the elution of AMB, whereas the colour components were more rapidly eluted. Tetrahydrofuran reduced the retention time of AMB only. On the basis of these results, the volume ratio of the four solvents (acetonitrile, methanol, tetrahydrofuran and 10 m*M* acetate buffer pH 7.0) was examined to find the best mobile phase for the present HPLC analysis. As shown in Table III and Fig. 1c, the acetonitrile–methanol–tetrahydrofuran–10 m*M* acetate buffer (pH 7.0) ratio of

TABLE II

EFFECT ON AMB RECOVERY OF EVAPORATION TO DRYNESS OF THE METHANOL ELUENT FROM SEP-PAK C_{18} CARTRIDGE UNDER AIR OR NITROGEN

Sample solution (25 ml, 200 ng/ml of pooled human urine) was pre-treated using a Sep-Pak C_{18} cartridge, then the methanol eluate was evaporated to dryness under a stream of air or nitrogen at 65 °C. The residue was redissolved in 0.5 ml of methanol, and the AMB concentration was determined by HPLC method.

	n	Found (ng/ml)	Recovery (%)
Air	1	124.6	62.3
	2	203.2	101.6
	3	203.2	101.6
	4	136.2	68.1
	5	115.4	57.7
Mean ± S.D.		156.5 ± 43.3	78.3 ± 21.6
C.V. (%)		27.6	27.6
Nitrogen	1	197.0	98.5
	2	201.6	100.8
	3	197.0	98.5
	4	199.4	99.7
Mean ± S.D.		198.8 ± 2.2	99.4 ± 1.1
C.V. (%)		1.1	1.1

TABLE III

EFFECTS OF CONSTITUTION OF THE MOBILE PHASE ON RETENTION TIMES OF AMB AND URINE COLOUR COMPONENTS

The constitution of the mobile phase is presented as the volume ratio of each solvent. HPLC conditions except for the mobile phase were as decribed in Experimental. The urine sample was prepared from pooled human urine, and the standard solution (200 ng/ml AMB) was used for the experiment.

Line	Constitution of mobile phase (ratio of volume)				Retention time (min)	
	Acetonitrile	Methanol	Tetrahydrofuran	10 m*M* Acetate buffer (pH 7.0)	AMB	Colour components in urine
1	39	-	-	61	13.0	30
2	30	20	-	50	40.0	15
3	40	-	12.5	60	4.5	20
4	30	20	10	40	4.0	7
5	30	20	5	45	8.0	8
6	35	15	5	45	6.0	9
7	25	20	5	50	14.5	12

TABLE IV

WITHIN-RUN PRECISION AND RECOVERIES FOR DETERMINATION OF URINE AMB BY HPLC METHOD

Sample solutions used for the precision study were as described in Experimental (calibration curve).

Added (ng/ml)	Found (mean ± S.D.) (ng/ml)	C.V. (%)	Recovery (%)	*n*
0.5	0.5 ± 0.05	10.4	95.4	4
1.0	1.1 ± 0.10	8.9	112.4	4
4.0	3.5 ± 0.13	3.8	87.6	5
8.0	7.5 ± 0.19	2.5	93.6	5

TABLE V

URINARY EXCRETION OF ORALLY ADMINISTERED AMB IN PATIENTS

Serum AMB levels were from our previous report [11]. Total serum AMB level was calculated using 3 l as the volume of body-circulating plasma.

Subject	Urine AMB (μg per day)				
	Day 1	Day 2	Day 3	Day 4	Day 5
A	0.1	-	1.2	-	0.6
B	15.5	9.0	28.1	14.3	28.1
C	8.8	45.0	11.5	3.0	-
D	1.0	-	6.5	2.8	16.2
E	14.6	20.3	17.6	41.8	48.2
F	2.0	3.9	3.7	7.8	-
Mean ± S.D.	7.0 ± 6.9	19.6 ± 18.3	11.4 ± 10.0	13.9 ± 16.3	23.3 ± 20.1
Percentage of dose, $\times 10^3$	0.14	0.41	0.24	0.29	0.49
Mean serum level (μg/ml)	0.05	0.08	0.095	0.10	0.10
Total serum AMB (μg)	150	240	285	300	300
Percentage of dose, $\times 10^3$	3.1	5.0	5.9	6.3	6.3
Urinary excretion rate (urine/serum level × 100%)	4.7	8.2	4.0	4.6	7.8

25:20:5:50 (v/v) gave the best chromatogram; the elution of colour components was complete by 12 min, and the retention time of AMB was 14.5 min.

The internal standard (*p*-nitrophenol) used previously for HPLC analysis of the serum AMB [11] was unsuitable for the present method because *p*-nitrophenol overlapped with the urine colour components in the chromatogram. Although many compounds that have a similar spectrum to that of AMB were examined, we found none that was suitable. In the present study, methanol solution containing 200 ng/ml AMB was used as a control for estimating the AMB

level from the peak height in the chromatogram. In the present HPLC method, the calibration curve for the determination of urine AMB gave a good linearity [$y = 10.0x - 1.8$; coefficient of correlation (r) = 0.9995] over the range of 0.5–8.0 ng/ml AMB, and the lower limit of determination was estimated to be 0.5 ng/ml AMB in urine, based on a peak height corresponding to twice the baseline noise.

Within-run precision and recoveries were determined for urine samples containing 0.5–8.0 ng/ml AMB (Table IV). The C.V. was 2.5–8.9% at 1.0–8.0 ng/ml ($n = 4$–5), whereas at 0.5 ng/ml it was 10.4%.

Our method was used to obtain the daily profile of urinary AMB excretion in six diabetic patients with urinary fungal infections (*Candida albicans*) who received AMB (Fungizone, 4800 mg per day) orally for five successive days. As shown in Table V, the urinary AMB excretion was very low (7.0–23.3 μg per day, $0.14–0.49 \cdot 10^{-3}$% of dose) with large inter-subject variations, and the urinary excretion rate (urine level/serum level × 100%) was 4.0–8.2%, which was slightly higher than the result of Atkinson and Bennett [16] (ca. 3%) following intravenous (i.v.) administration.

In our previous study [11], the total serum AMB level was 150–300 μg ($3.1–6.3 \cdot 10^{-3}$% of dose) in patients who received AMB (4800 mg per day) orally. Even immediately after completion of i.v. infusion of 50 mg of AMB (administered over a period of several hours), the average peak serum levels were ca. 2 μg/ml (total serum AMB level, 6 mg; ca. 10% of dose) [17]. Thus, low urinary AMB excretion suggests that a large amount of the orally administered drug is not absorbed by the gut and that drug decomposition occurs in vitro together with its metabolism and biliary excretion. Renal excretion appears to be a relatively minor pathway for the elimination of AMB.

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