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New Application Method of Digitalis Glycoside Measurement in Human Urine by Fluorometry

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Summary

A simple and sensitive fluorometric determination of digitoxin in human urine is described. The reaction was based on the formation of a fluorophore on heating digitoxin with 90 per cent sulfuric acid at 70°C for 15 min. The fluorescence emission occurred at 435 nm on excitation at 405 nm. The linear range of the method was 10-100 pmol/ml of digitoxin. The coefficient of variation was 6.4 per cent (n=7) when 100 pmol/ml of digitoxin were used. The detection limit of urinary digitoxin was 10 pmol/ml (S/N=2).

Plasma digitalis glycoside levels in the patients treated with these drugs are very low even during their use as therapeutic drugs for cardiac stimulant (0.8-25 ng/ml) (1). Although digitalis glycosides are available in many forms, the purified glycosides, digoxin (12-hydroxydigitoxin) and digitoxin, are the most widely used preparations in the world.

These drugs are metabolized very slowly in the body, and the half-life is estimated at 1 to 6 days (2). The volume and the half-life of unchanged forms of these drugs and the metabolites excreted into the urine are associated with renal function, i.e., the half-life of these drugs or derivatives are prolonged in patients with renal insufficiency (3). Therefore, it is very important for adequate treatment with digitalis glycosides to monitor renal function by sensitively measuring the unchanged urinary forms of these drugs in order to protect against side effects.

Most previously reported methods for the determination of digitalis glycoside concentrations in serum or urine are radio-immunoassay (4) and enzyme immuno assay (5). Physico-chemical methods like TLC (Jeliffe (6)) or HPLC (Nachtmann (7)) are still behind the above-mentioned major methods because of their

incomplete application for serum or urine samples.

Another basic method was also reported (8) which focused on the fluorescence production by strong acid treatment with HCl or trichloroacetic acid solutions with digitalis glycosides, and the digitalis glycosides were isolated and quantified thereafter.

The present report focuses on the digitoxin-originated strong fluorescence due to the reaction with sulfuric acid solution, and demonstrates the measurement of digitoxin in human urine by a fluorometric analysis in order to simplify and improve previous methods.

Methods

Materials and Apparatus

Digitoxin was purchased from a commercial source (Sigma Chemical Co., Ltd., St Louis, Mo., USA). Aqueous sulfuric acid solutions were prepared with concentrated sulfuric acid (97 per cent, special grade, Wako Pure Chemical Industries, Ltd., Osaka, Japan). All other chemicals were of reagent grade, and deionized and distilled water was used throughout.

Absorption and fluorescence spectra were measured with a Hitachi F-4000

TABLE 1. *Analytical Procedure for Digitoxin in Urine*

Urine containing digitoxin 1 ml
↓ Saturated NaCl solution 1 ml, H_2O 5 ml, Acetic acid 1 drop
Heated at 95°C for 10 min in the boiling water bath
↓
Neutralized with 10% Na_2CO_3 solution
↓
Extracted with chloroform (10 ml × 2)
↓
Evaporated organic phase under reduced pressure
↓
Residue
↓ 90% H_2SO_4 10 ml
Heated at 70°C for 15 min
↓
Cooled to room temperature
↓
Fluorescence measurement (Ex. 405 nm, Em. 435 nm)

fluorescence spectrophotometer.

Calibration Procedure

Stock solutions of digitoxin were prepared in ethanol at the concentration of 10^{-3}M, and diluted with distilled water to make up standard solutions. (Table 1) Urine samples from healthy subjects were deproteinized as reported earlier (9), i.e., one ml of saturated NaCl solution, 5 ml of distilled water and 1 drop of acetic acid were added to the samples and heated in a boiling water bath for 10 min., cooled, and subsequently followed by neutralization with 10 per cent Na_2CO_3 solution. After the deproteinization, the sample solutions were extracted twice with 10 ml of chloroform, and the chloroform layer was evaporated under a vacuum. The residue was dissolved in 10 ml of 90 per cent of H_2SO_4 solution, and heated at 70°C for 15 min. to complete the reaction. After cooling at room temperature, fluorescence intensity was measured (Ex.: 405 nm, Em: 435 nm) using 1.0×10^{-5}M quinine in 0.1N H_2SO_4 as a standard.

Results and Discussion

Determination of Reaction Condition of Digitoxin with H_2SO_4 Solution

As shown in Fig. 1, digitoxin is composed of three sugars at the 3-0-position and an unsaturated lactone ring at the 14-position of the steroid nucleus. Digitoxin in H_2SO_4 solution showed maximum absorption (λmax) at 220 nm at room temperature (Fig. 2). In contrast, the spectrum showed a widely-ranged profile after the heat treatment with H_2SO_4 solution, ranging from the ultra-violet region to the visible region. This change is probably attributable to the formation of anhydrous derivatives which are produced by the reaction with mineral acid, or other various reaction products which are difficult to isolate. (10), (11)

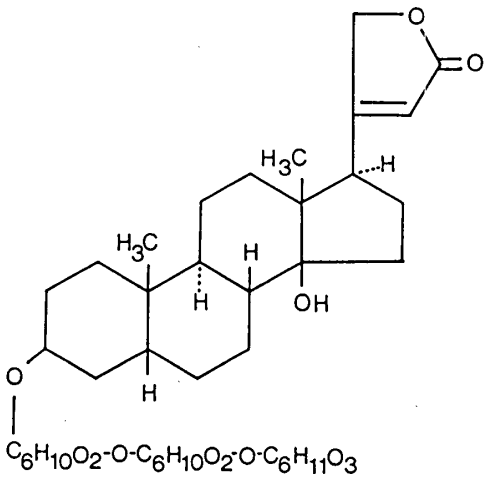

FIG. 1. Digitoxin

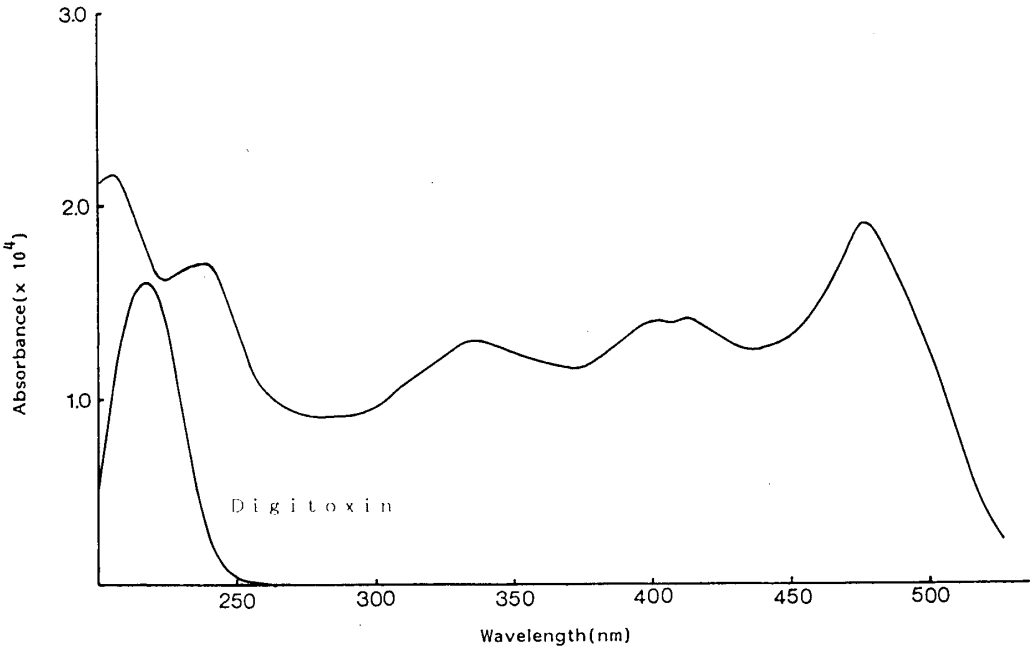

FIG. 2. UV spectrum for digitoxin treated at 70°C for 15 min in 90% H_2SO_4

As shown in Fig. 3, the spectrum of fluorescence intensity was seen in the maximum fluorescence at 435 nm (Em) when excited at 405 nm (optimal excitation wave length ; Ex).

In order to determine the optimum analytical condition for digitoxin quantification, the concentration effect of H_2SO_4 on fluorescence production was determined at 70°C using a time-course study.

The fluorescence due to the digitoxin reaction in the 90 per cent H_2SO_4 solution showed a higher intensity than the others (80 per cent, 70 per cent, and 60 per cent H_2SO_4 solutions), and reached a plateau level in only 10 min. (Fig. 4). A gradual decrease in intensity followed. The solution treated with concentrated H_2SO_4 showed the same fluorescence intensity as 80 per cent H_2SO_4 solution though the reason is unknown. Temperature effect on fluorescence production by digitoxin in 90 per cent H_2SO_4 is shown in Fig. 5. This result indicates that the best condition of this reaction for stable and maximal fluorescence intensity is 70°C for 15 min. However, we confirmed the stability of the cooled sample up to 120 min.

Application Method for Urine Samples

In order to quantify urinary digitoxin level, the application method of this reaction was investigated. Urine samples which contain known amount of digitoxin from healthy subjects were prepared as described above ; i.e., after the deproteinization of urine, digitoxin was extracted with chloroform. Recovery percentage of digitoxin after the extraction was 98.5 per cent, and it was confirmed

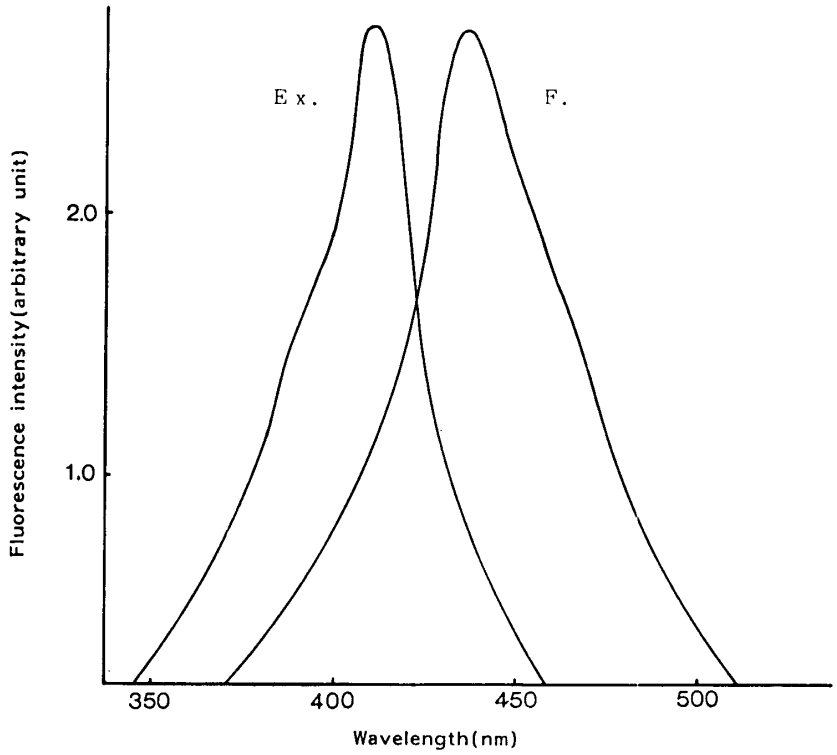

FIG. 3. Excitation and emission spectra for digitoxin treated at 70°C for 15 min in 90% H_2SO_4

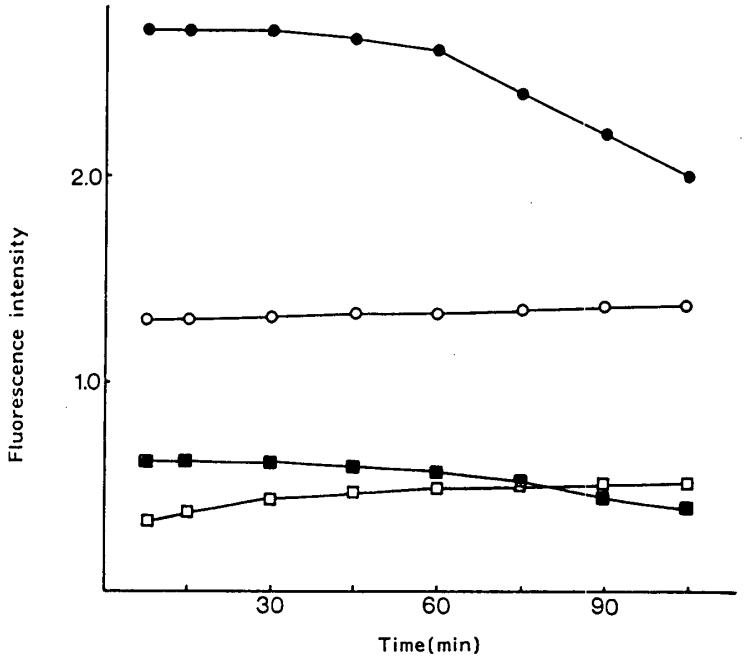

FIG. 4. Concentration effect of H_2SO_4 on the fluorescence production of digitoxin at 70°C

●: 90% H_2SO_4, ○: 80% H_2SO_4, ■: 70% H_2SO_4, □: 60% H_2SO_4.

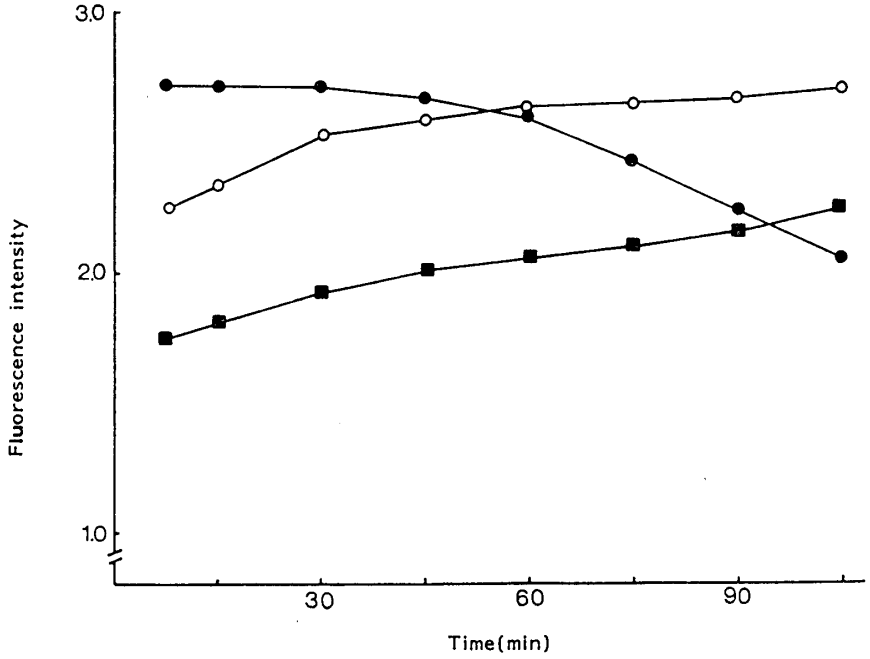

FIG. 5. Temperature effect on the fluorescence production of digitoxin in 90% H_2SO_4
●: 70°C, ○: 50°C, ■: room temperature.

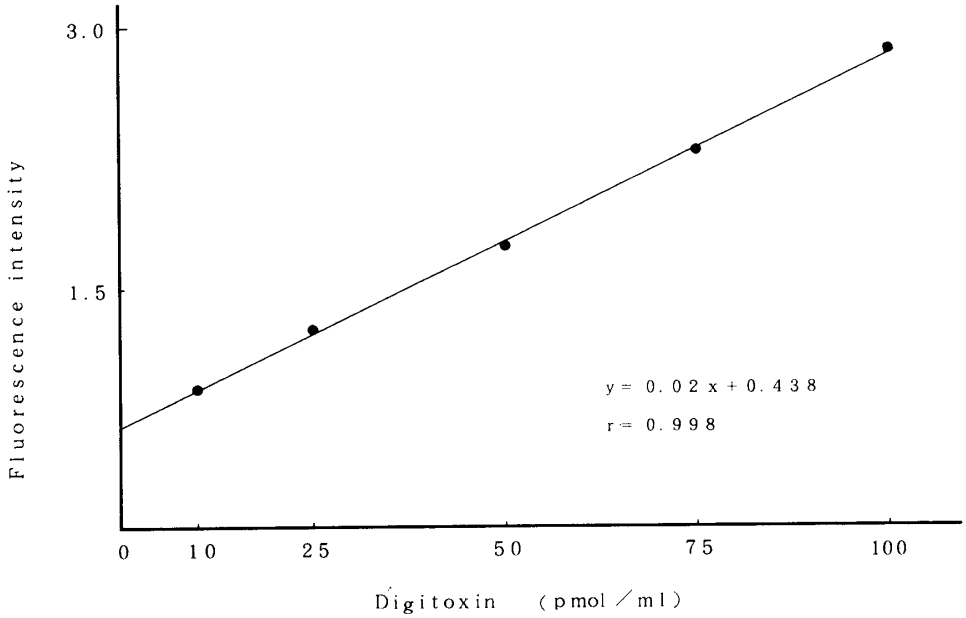

FIG. 6. Working curve for digitoxin in urine by proposed method

that this percentage exceeded that of other solvents like dichloromethane, dichloroethane, benzene, cyclohexane, acetonitrile and ethyl acetate. The chloroform extract was then evaporated to dryness under a vacuum. Followed by addition of 90 per cent H_2SO_4 solution, the fluorescence intensity of the reacted solution

was measured (Ex : 405 nm, Em : 435 nm).
A calibration curve (Fig. 6) was made by using these samples, and a good linear correlation was observed between 10 and 100 pmol/ml of digitoxin ($Y=0.020X+0.438$, $r=0.998$). The coefficient of variation was 6.4 per cent ($n=7$) when 100 pmol/ml of digitoxin were used, which indicates enough reproducibility to do this routinely. The detectable limit of urinary digitoxin was 10 pmol/ml (S/N ratio = 2).

It is already known that digitoxin is effective when its plasma level is around at 15 to 25 ng/ml, and that almost 33 per cent of the unchanged form of the drug is excreted into the urine (12). Therefore, human urine has enough digitoxin to quantify the amount of unchanged form of the drug from the result of the dose-fluorescence intensity curve. We are now trying to apply this method to urine of actual patients who are treated with digitoxin as a cardiac stimulant.

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