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Short communication

Liquid chromatographic determination of unbound flecainide in therapeutic drug monitoring

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Abstract

An assay method was developed for determining unbound flecainide in serum by reversed phase-high performance liquid chromatography (HPLC). Serum water including unbound flecainide was separated by ultrafiltration of the serum sample and subjected to C_{18}-cartridge extraction followed by HPLC analysis. The recovery of flecainide from serum water was greater than 93%. The coefficient variations for intra- and inter-day assay of flecainide were smaller than 2.4 and 3.7%, respectively. We applied the method to determining unbound flecainide in serum samples collected from 20 patients receiving oral flecainide (150–300 mg/day) for tachyarrhythmia. Total and unbound concentrations for serum flecainide were 403.5 ± 200.8 ng/ml and 180.2 ± 95.0 ng/ml, respectively. Linear relationship was observed between total and unbound concentrations ($r = 0.978$, $P < 0.0001$). Percent unbound ($44.3 \pm 5.7\%$) determined in the present study agreed with the reported values. The percentage unbound tended to increase in the samples with lower α_1-acid glycoprotein (< 60 mg/dl). The assay method can be applied to routine determination of unbound flecainide in therapeutic drug monitoring.

Keywords: Flecainide; Unbound fraction; α_1-Acid glycoprotein; Therapeutic drug monitoring

1. Introduction

Protein binding can affect the pharmacokinetic processes of drug distribution and elimination [1–5]. Flecainide (Fig. 1), Vaughan Williams class 1c antiarrythmic agent, is known to bind α_1-acid glycoprotein (AAG) and albumin in serum [6]. Previous studies revealed that 40–60% of flecai-

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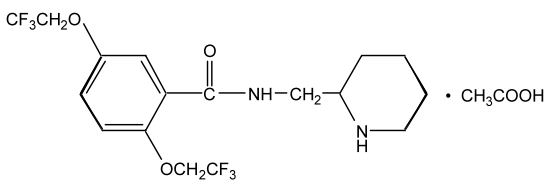
(a) Flecainide acetate



(b) Internal standard

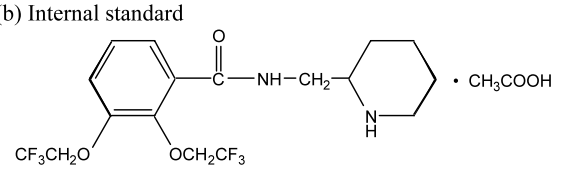


Fig. 1. Chemical structures of flecainide acetate (a) and internal standard [*N*-(2-piperidylmethyl)-2,3-bis (2,2,2-trifluoroethoxy) benzamide acetate] (b).

nide circulating in blood was unbound [7–9]. Since unbound fraction for serum flecainide is relatively high, change in the fraction might affect the therapeutic efficacy of flecainide, which required the serum concentration monitoring for making dosage regimen. It, therefore, is interesting for predicting therapeutic implications to determine unbound flecainide as well as the total concentration in serum [9].

The unbound flecainide has been determined by equilibrium dialysis followed by ^{14}C-flecainide dilution technique [7] or high performance liquid chromatography (HPLC) [10]. In the present study, we developed an assay method for determining unbound flecainide using an ultrafiltration technique prior to solid phase extraction and HPLC analysis. We applied this method to routine monitoring of unbound flecainide and examined the effects of AAG levels on the unbound flecainide in patients with tachyarrhythmia.

2. Methods

2.1. Chemicals and instruments

Flecainide acetate and the internal standard [*N*-(2-piperidylmethyl)-2,3-bis (2,2,2-trifluoroethoxy) benzamide acetate] (Fig. 1) were supplied by Eisai Co. (Tokyo, Japan). 1-Pentanesulfonic acid sodium salt was purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of HPLC or analytical reagent grade. C_{18}-Cartridges (Extract-Clean C_{18}, 100 mg) used for solid phase extraction and 12-port Manifold were obtained from Alltech Associates, Inc. (IL, USA). Ultrafiltration filter units (MINICENT-10) were purchased from TOSOH (Tokyo, Japan). This filter unit cut-off the protein over the molecular weight of 10 000.

2.2. Preparation of solutions

The control serum was prepared using an alternative human serum (Twin-consera H, Nissui, Tokyo, Japan). Serum water was separated from the serum by ultrafiltration. The serum water samples spiked with flecainide acetate at concentrations of 62.5, 125, 250 and 500 ng/ml as flecainide were used as the standard specimens for calibration and stored at −20 °C until use. The internal standard was prepared as the 5 μg/ml solution in distilled water and stored at 4 °C until analysis.

2.3. HPLC apparatus and analytical conditions

HPLC system used in the present study consisted of pump (CCPD, TOSOH, Tokyo, Japan), UV detector (UV-8010, TOSOH) and integrator (C–R4A, Shimadzu, Kyoto, Japan). ODS column (TSK-GEL, 4.6 i.d. × 250 mm, TOSOH) for HPLC system was maintained at room temperature. The detection wavelength was set at 298 nm. The mobile phase solution, consisted of 0.1 M 1-pentanesulfonic acid sodium salt, acetonitrile and acetic acid (250:206:2.5 v/v), was pumped at a flow rate of 1.0 ml/min.

2.4. Assay procedures

For determination of unbound flecainide, serum water was separated from 0.5 ml of serum using an ultrafiltration filter units with centrifugation at 37 °C, 3000 × *g* for 60 min. Two-hundred and fifty microliter aliquot of serum water was added with 75 μl of internal standard solution and then, was

alkalinized by adding 50 μl 0.02 M Na_2CO_3. The mixture was loaded onto C_{18}-cartrige pretreated with 1 ml methanol and followed by 1 ml distilled water. The C_{18}-cartrige was set at Manifold and subsequently washed with 1 ml water, and 1 ml 50% methanol under the vacuum. Flecainide and internal standard were eluted by treating the cartridge with 1 ml methanol and were collected into glass tubes. The effluents were placed in a dry thermo bath (45 °C) and evaporated to dryness under nitrogen gas flow. The sample was reconstituted with 50 μl mobile phase solution and the 20 μl aliquot was injected into the column.

Serum concentration of total flecainide was determined by HPLC previously described by us [11]. Serum AAG concentrations were measured by radial immunodiffusion kit (MBL plate®, Medical and Biological Laboratories Corp., Nagoya, Japan).

2.5. Patients

Twenty out-patients (16 males and 4 females) receiving oral flecainide for tachyarrhythmia were enrolled for this study. Patient characteristics were presented on Table 1. The liver and kidney functions for all patients were normal. Seven patients received other antiarrhythmic medications, propranolol, atenolol and carteolol, which were possible drugs being bound to AAG [12,13]. Blood drawing was carried out at 9:00–11:00 a.m. in out-patients visit. Patients postponed taking morning flecainide until blood drawing on that day. Informed consent was obtained from all patients and the study was approved by The Ethical Committee of the University of Tsukuba.

Table 1
Patients' profile

Age (yr)	58.5±13.1
Sex (M/F)	16/4
Weight (kg)	66.5±14.6
Diagnosis (n)	
Atrial fibrillation	6
Atrial fibrillation and atrial flutter	5
Atrial flutter	4
Paroxysmal supraventricular tachycardia	2
Wolff–Parkinson–White syndrome	2
Supraventricular premature contractions	1
Flecainide acetate dose (mg/day)	202.5±30.2
Liver and kidney function	
Aspartate aminotransferase (IU/l)	21.6±4.4
Alanine aminotransferase (IU/l)	19.5±7.9
Serum creatinine (mg/dl)	0.74±0.13
Blood urea nitrogen (mg/dl)	16.2±4.6

2.6. Statistical analysis

All data were presented as the mean±S.D. Statistical analysis of the data for the serum flecainide concentrations and AAG levels were examined using unpaired Student's *t*-test. The *P* values less than 0.05 were considered to be statistically significant.

3. Results

Typical chromatogram for determining flecainide in serum water (unbound) was indicated in Fig. 2. The chromatogram was almost the same with that of serum for determining total flecainide previously described [11]. No interfering peak was observed for detecting flecainide and internal standard in serum water separated from patients' serum. The detection limit of flecainide in serum water was as low as 25 ng/ml.

The regression curve for determining flecainide in serum water was linear at the concentration range of 62.5–500 ng/ml. The equation of the line calculated by regression analysis for flecainide was

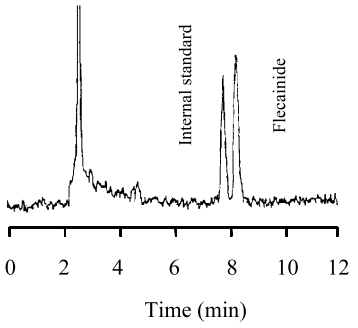


Fig. 2. Typical chromatogram for determination of flecainide in serum water separated from control serum. The concentration for unbound flecainide was 517 ng/ml.

$Y = 485.48X - 31.26$ ($r = 0.9999$), where Y was the serum concentration in the serum water (ng/ml) and X, the peak height ratio of flecainide to internal standard.

The recoveries of flecainide from serum water examined at concentrations of 80 and 400 ng/ml were 93.8±3.8% and 95.1±2.4%, respectively, with the coefficient variation (CV) values less than 4.0%. The intra- and inter-day precision evaluated at 80 and 400 ng/ml were 1.9–2.4% and 3.6–3.7%, respectively (Table 2). The relative errors (bias) for intra- and inter-day assay were less than 2.2 and 1.6%, respectively.

Total and unbound concentrations of serum flecainide were 403.5±200.8 ng/ml (range 163–1004) and 180.2±95.0 ng/ml (range 73–442), respectively in 20 patients receiving 202.5±30.2 mg/kg flecainide acetate daily (Table 3). Unbound fraction (% unbound) for flecainide was 44.3±5.7% (range 34.1–57.8). Serum AAG concentrations for all serum samples were in normal range (63.0±16.0 mg/dl, range 44.4–114.8). A significant difference in percentage of unbound drug was observed between the two sample groups, AAG levels <60 mg/dl and $\geqq 60$ mg/dl. The percent unbound significantly increased in the samples with <60 mg/dl compared with those with $\geqq 60$ mg/dl (47.4±5.85 vs. 41.8±4.36%, $P < 0.05$) (Table 3). There was no significant difference in albumin between two sample groups (4.0±0.1 vs. 4.0±0.4 g/dl).

The positive correlation was found between unbound and total concentrations of flecainide ($r = 0.978$, $P < 0.0001$, Fig. 3). Eight patients complained of palpitation, whose total and unbound concentrations were 268.3±82.1 ng/ml and 115.2±32.2 ng/ml, respectively. These levels were significantly lower than those in patients without palpitation (493.7±207.7 ng/ml and 223.5±99.1 ng/ml, $P < 0.01$) (Fig. 3).

4. Discussion

We used ultrafiltration instead of equilibrium dialysis for separating unbound flecainide from serum samples. Since ultrafiltration can separate serum water without diluting the samples, we could apply the HPLC to determining unbound flecainide above the detection limit of 25 ng/ml. Our method employing solid phase extraction and HPLC equipped with ordinary ODS column and UV detector showed a sufficient sensitivity for monitoring unbound flecainide in the therapeutic range of 200–1000 ng/ml for the total concentration. Assay precision confirmed in the intra- and inter-day validations was almost the same with those for total concentration. The most important advantage for the present method is to use same HPLC system with that for determination of total flecainide. This is very convenient for routine therapeutic drug monitoring of this drug in the hospital and clinical institutions.

The concentration for unbound flecainide was 180.2±95.0 ng/ml (range 73–442) in the samples with total concentration of 403.5±200.8 ng/ml (range 163–1004). The percentage unbound flecainide was 44.3±5.7% (range 34.1–57.8), which agreed with previous studies (40–60%) conducted by equilibrium dialysis: Caplin et al.; 39±10% [7], Zordan et al.; 59±6% [8], and Padrini et al.; 56.9±4.9% [9]. This observation suggested that the present assay method using ultrafiltration was

Table 2
Intra- and inter-day precision for determination of flecainide

Concentration (ng/ml)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
	Mean±S.D. (ng/ml)	CV (%)	Bias (%)	Mean±S.D. (ng/ml)	CV (%)	Bias (%)
80	81.8±1.9	2.4	2.2	80.1±2.9	3.7	0.1
400	393.5±7.6	1.9	1.6	393.7±14.3	3.6	1.6

For intra-day precision, five sets of each control sample were assayed on the same day. For inter-day assay precision, five sets of each control sample were assayed on 5 different days.

Table 3
Comparison of total concentration, unbound concentration and percent unbound of flecainide between high and low AAG groups

	AAG (mg/dl)	Flecainide concentration (ng/ml)		
		Total	Unbound	% Unbound
Low AAG (<60, $n=9$)	51.6±5.4]*	367.7±117.8	175.2±64.0	47.4±5.9]*
High AAG ($\geqq 60$, $n=11$)	72.3±15.9	433.1±251.8	184.3±117.7	41.8±4.4
All patients ($n=20$)	63.0±16.0	403.5±200.8	180.2±95.0	44.3±5.7

* Significant difference was observed, $P<0.05$.

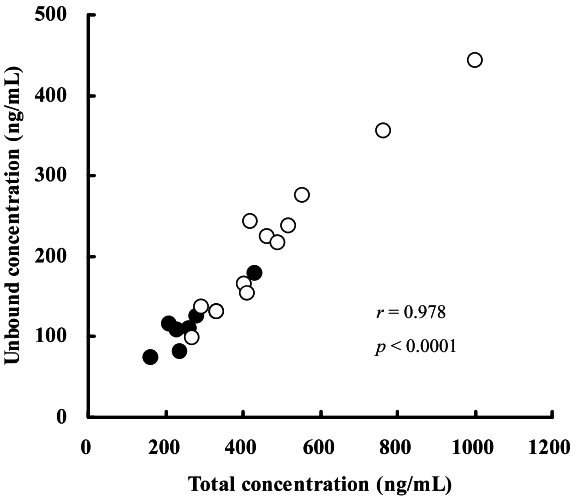


Fig. 3. Relationship between total and unbound concentration of flecainide in patients with (●) and without (○) palpitation.

almost equal to previous ones using equilibrium dialysis in determining unbound flecainide.

Serum AAG levels, but not albumin, both of which bind to flecainide, affected the unbound fraction. Percent unbound increased in the samples with lower AAG (<60 mg/dl) compared with those of higher AAG ($\geqq 60$ mg/dl). Correlation between unbound flecainide and serum AAG levels is controversial. Our observations agreed with the report of Johnston et al. [6] but disagreed with that of Caplin et al. [7]. Caplin et al. examined binding of flecainide to serum AAG in in vitro experiments and found that unbound flecainide increased in accordance with elevation of serum AAG in patients with myocardial infarction. The discrepancy between the present and Caplin's results might be due to the difference in in vivo and in vitro experiments and in the AAG levels, which were higher in the Caplin's study (104±39 mg/dl) compared with us (63.0±16.0 mg/dl). Our data showed for the first time that AAG affected unbound fraction of flecainide in vivo in patients with tachyarrhythmia. Further study in the patients with wide range of serum AAG levels will be required to confirm the effects of AAG on the unbound flecainide.

Significant difference in unbound flecainide was observed between the patients with and without palpitation. The patients without palpitation had higher unbound flecainide as well as the total serum concentration. The lower limit of therapeutic range for unbound flecainide might be around 150 ng/ml (Fig. 3). The present results and Padrini's findings observing a linear relationship between unbound flecainide and QRS enlargement [9] suggest some merits for determining unbound flecainide to predict the pharmacodynamics of flecainide. The impact of determining unbound flecainide in the TDM should be clarified in the future studies.

5. Conclusions

The assay method developed in the present study was applied to determining unbound flecainide concentration in the patients with tachyarrhythmia. The percentage of unbound fraction for flecainide was 44.3±5.7%, which agreed with reported value. It was also found that the percent unbound tended to increase in the samples with lower AAG. The present assay method can be utilized in further clinical studies for assessing the impact of unbound flecainide in the TDM.

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