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Note

Determination of β-methyldigoxin and its metabolites by high-performance liquid chromatography and fluorescence polarization immunoassay

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β-Methyldigoxin (β-MD) is a cardiac glycoside which is methylated at the 4-position of the terminal digitoxose unit to improve the gastrointestinal absorption of digoxin. β-MD is metabolized in the body into digoxin (D3), digoxigenin bisdigitoxoside (D2), digoxigenin monodigitoxoside (D1) and digoxigenin (D0). The absorption rate of β-MD is about 70–100% [1–5] and that of digoxin is about 65–80% [6–8], and both of them have the same pharmacological effects on the heart [9]. However, the pharmacokinetics of β-MD are not well known because the concentrations of β-MD and its metabolites in biological fluids are low (ng/ml range). Rietbrock et al. [2] and Hinderling et al. [10] reported their assay in biological materials using thin-layer chromatography (TLC), but the methods were time-consuming. Another method involving radioimmunoassay (RIA) combined with high-performance liquid chromatography (HPLC) was developed by Malini et al. [11]. HPLC is useful for separating cardiac glycosides easily and RIA is a sensitive method that has commonly been used for their determination. Recently, fluorescence polarization immunoassay (FPIA) has been applied to the determination of the concentration of digoxin in plasma or serum; it has a similar sensitivity to RIA and does not require radioactive materials. However, the specificity of FPIA is limited, like RIA, because antibodies may cross-react to varying extents with all of the hydrolysed metabolites and other substances such as spironolactone [12].

We have developed a sensitive and specific HPLC–FPIA method for the determination of β-MD and its metabolites in plasma and urine.

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EXPERIMENTAL

Materials

All solvents used for extraction and chromatography were of HPLC grade from Wako (Osaka, Japan). β-MD, D3, D2, D1 and D0 were obtained from Boehringer Mannheim (Mannheim, F.R.G.). A Chem Elut column (column part No. 1001 for plasma, No. 1005 for urine) was purchased from Analytichem International (Harbor City, CA, U.S.A.).

Cross-reactivity

Cross-reactivities were tested with β-MD, D2, D1 and D0 by spiking blank plasma with each compound in the range 0.5–5.0 ng/ml and then assaying these samples using a TDx Digoxin II Assay kit for FPIA (Dainabot, Tokyo, Japan). Percentage cross-reactivity = 100 × [concentration measured as digoxin by TDx analyser (TDx value)/concentration of each compound].

Extraction

Plasma (1 ml) was applied to a Chem Elut column and after 3 min the column was eluted with 20 ml of dichloromethane. This eluate was evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 200 μl of *n*-heptane–isopropyl alcohol–water (69:30:1) instead of the HPLC mobile phase because the volume of the sample reconstituted with the mobile phase could easily change during mixing. A volume of 100 μl was injected on to the HPLC column.

Urine (5 ml) was also extracted with 30 ml of dichloromethane using a Chem Elut column, the remainder of the procedure being identical with that described above for plasma.

Chromatography

The HPLC system consisted of a CCPM dual pump, an AS-8000 automatic sampler with a 100-μl loop and a UV-8000 UV detector (220 nm) (all from Tosoh, Tokyo, Japan). The column was Wakopak LiChrosorb Si 60 (150 mm × 4.6 mm I.D.) of 5 μm particle size (Wako). The mobile phase was *n*-heptane–isopropyl alcohol–dichloromethane–water (69:20:10:1) at a flow-rate of 1.5 ml/min. Each fraction eluted was collected according to the retention time, which was decided by previously injecting a standard mixture (1 μg/ml) because it was impossible to detect β-MD and its metabolites in the ng/ml range spectrophotometrically. The fraction collector used in this study was an FC-8000 (Tosoh).

Fluorescence polarization immunoassay

The fractions of eluate corresponding to β-MD and its metabolites were evaporated under a stream of nitrogen. The residues were reconstituted with 0.6 ml of supernatant solution obtained previously from blank plasma deproteinized with the precipitation reagent from the TDx Digoxin II Assay kit and measured by the TDx analyser.

Analyses

The within-run precision was assessed using five separate standard samples prepared from blank plasma or urine to which β-MD and its metabolites had been added at concentrations of 0.5 and 3.0 ng/ml. For the day-to-day precision, the same concentrations of standard plasma and urine samples were analysed on four separate days. The extraction recovery of each compound was also assessed at levels of 0.5 and 3.0 ng/ml.

Five spiked plasma or urine samples were determined according to the assay procedure. These TDx values from plasma samples were compared with the values obtained from the direct TDx analysis of unextracted plasma samples. As urine samples could not be assayed by TDx directly, the TDx values for urine samples were also compared with those of unextracted plasma samples to determine the recoveries from urine samples.

Calibration graphs were established in the ranges 0.2–5.0 ng/ml (β-MD, D3), 0.1–5.0 ng/ml (D1) and 0.05–5.0 ng/ml (D1, D0) for plasma and 0.02–50 ng/ml (D0, D1, D2, D3 and β-MD) for urine. Calibration samples were assayed in duplicate. The equation of the regression line was calculated as $y=ax+b$, where x is the concentration of each compound and y is the TDx value. The measurable concentration in the TDx Digoxin II Assay kit is less than 5.0 ng/ml as digoxin. Therefore, if the reconstituted sample after HPLC separation indicated a TDx value higher than 5.0, the sample was diluted appropriately with the supernatant solution described previously and measured by TDx again. The TDx value of the sample was calculated by multiplying the value derived from TDx by the appropriate dilution factor.

RESULTS AND DISCUSSION

Cross-reactivity

Significant cross-reactivities were found for β-MD and its metabolites. The mean cross-reactivities of β-MD, D2, D1 and D0 were 86.3, 147.0, 164.0 and 183.0%, respectively, as shown in Table I. It was impossible to determine the percentage cross-reactivity of D0 at 3 and 5 ng/ml and D1 and D2 at 5 ng/ml

TABLE I

CROSS-REACTIVITIES OF β-METHYLDIGOXIN AND ITS METABOLITES AT SEVERAL CONCENTRATIONS IN FPIA

Concentration (ng/ml)	Cross-reactivity (mean ± S.D., $n=5$) (%)			
	β-MD	D2	D1	D0
0.5	96.0 ± 4.24	156.0 ± 5.48	170.8 ± 4.60	192.0 ± 11.23
1.0	87.0 ± 3.16	143.2 ± 4.97	158.2 ± 3.03	184.0 ± 4.69
2.0	81.1 ± 1.98	146.4 ± 2.58	164.3 ± 4.96	173.0 ± 4.50
3.0	82.2 ± 2.88	142.4 ± 2.83	142.4 ± 2.83	–
5.0	85.8 ± 2.04	–	–	–
Overall mean ± S.D.	86.3 ± 6.05	147.0 ± 6.74	164.0 ± 5.86	183.0 ± 10.63

because these TDx values were above 5.0, which is the upper limit of the TDx Digoxin II Assay kit. Such high cross-reactivities prompted us to utilize FPIA for the determination of each compound following the HPLC separation.

Chromatography

For fractionation for FPIA, complete separation is required in order to avoid contamination of the metabolites. Eriksson et al. [13] reported a good separation of D3 and its metabolites by normal-phase HPLC with *n*-heptane–pentanol–acetonitrile–water. We examined this normal-phase system for the separation of β-MD and its metabolites, but β-MD, which was observed between D1 and D2, could not completely resolved. We therefore examined other mobile phase systems, and found that dichloromethane was a more efficient component for the separation for our purposes. Fig. 1 shows the standard chromatogram of β-MD and its metabolites. They were sufficiently resolved from each other using *n*-heptane–isopropyl alcohol–dichloromethane–water as the mobile phase. The retention times of D0, D1, β-MD, D2 and D3 were 8.0, 10.2, 12.4, 15.1 and 21.0 min, respectively, and their elution order was as reported by Eriksson et al. [13]. The normal-phase HPLC system offered the advantages of evaporating the collected eluate easily and avoiding the hydrolysis of β-MD to aglycone that can occur in a reversed-phase system.

Precision and recovery

The precision and recovery of the present method were sufficient for application to routine assays. Tables II and III give coefficients of variation (C.V.) for the determination of the each compound at two different concentrations and their

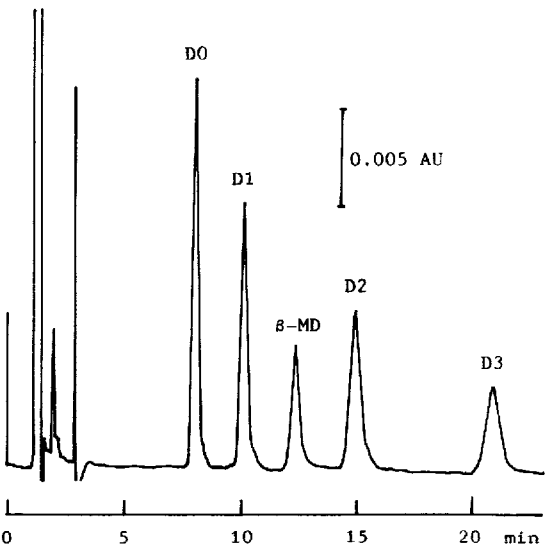

Fig. 1. Chromatogram of a standard mixture. Sample: 0.2 μg of each compound in 100 μl of the mobile phase. Peaks: D0 = digoxigenin; D1 = digoxigenin monodigitoxoside; β-MD = β-methyldigoxin; D2 = digoxigenin bisdigitoxoside; D3 = digoxin.

TABLE II

PRECISION AND RECOVERY IN THE DETERMINATION OF β-METHYLDIGOXIN AND ITS METABOLITES IN PLASMA

Compound	Concentration (ng/ml)	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)	
			Within-run ($n=5$)	Day-to-day ($n=4$)
β-MD	0.5	79.0 ± 4.53	5.73	9.08
	3.0	81.5 ± 2.81	3.45	4.76
D3	0.5	72.4 ± 5.56	7.68	14.52
	3.0	84.6 ± 2.70	3.20	6.52
D2	0.5	90.4 ± 4.40	4.86	2.36
	3.0	82.8 ± 2.08	2.51	6.52
D1	0.5	105.6 ± 5.43	5.14	4.14
	3.0	104.4 ± 3.26	3.12	4.78
D0	0.5	108.3 ± 5.33	4 92	3.69
	3.0	100.6 ± 2.42	2.41	7.43

TABLE III

PRECISION AND RECOVERY IN THE DETERMINATION OF β-METHYLDIGOXIN AND ITS METABOLITES IN URINE

Compound	Concentration (ng/ml)	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)	
			Within-run ($n=5$)	Day-to-day ($n=4$)
β-MD	0.5	102.4 ± 2.92	2.85	3.72
	3.0	99.6 ± 1.53	1.96	4 50
D3	0.5	90.9 ± 7.10	7 81	3.86
	3.0	92.8 ± 1.53	1.65	3.80
D2	0.5	102.5 ± 7.81	6.91	2.53
	3.0	98.8 ± 3.95	4.00	3.93
D1	0.5	94.0 + 5.47	5.82	3.19
	3.0	106.2 ± 2.48	2.34	2.75
D0	0.5	95 0 ± 2.67	2.80	0.84
	3.0	105.0 ± 2.72	2.59	2.40

recoveries for plasma and urine samples, respectively. For plasma samples, the within-run and the day-to-day C.V. ranged between 2.41 and 7.68% and 2.36 and 14.52%, respectively. Each recovery was more than 72%. For urine samples, the within-run C.V. was less than 7.81% and the day-to-day C.V. was less than 4.5%. The recoveries from urine were in the range 90.9–106%, which were better than those from plasma. In previous reports on the determination of digoxin and its metabolites, the recoveries from plasma were about 54–78% [14] and from urine about 70–90% [15,16]. As we used sorbent extraction with a Chem Elut column instead of liquid–liquid extraction, we could easily and efficiently extract β-MD and its metabolites from plasma and urine.

TABLE IV

CALIBRATION GRAPHS FOR β-METHYLDIGOXIN AND ITS METABOLITES

Regression equations: $y = ax + b$.

Compound	Plasma			Urine		
	a	*b*	Correlation coefficient	*a*	*b*	Correlation coefficient
β-MD	1.731	0 125	0.999	3.908	0.051	0.999
D3	2.366	0.252	0 996	3.822	0.154	1.000
D2	3.239	0.180	0.999	4.714	0.159	1.000
D1	3.973	0.106	0.999	5.984	0.135	1.000
D0	4.722	0.075	0.998	7.334	0.248	1.000

Linearity

The calibration graphs for β-MD and its metabolites in plasma and urine samples were linear over wide ranges. Each correlation coefficient was 0.996 or greater and each intercept was close to the origin. These ranges seemed sufficiently wide enough to determine these compounds in plasma or urine following the clinical use of β-MD. The slopes, intercepts and correlation coefficients are summarized in Table IV.

CONCLUSION

We have developed an HPLC–FPIA method for the determination of β-MD and its metabolites in plasma and urine. The method has the following advantages: the sensitivity is similar to that of RIA, radioactive materials are not required, the extraction time is short and the recoveries are improved by means of sorbent extraction with the Chem Elut column. This method is satisfactory for the determination of β-MD and its metabolites in plasma and urine and will be useful for more detailed pharmacokinetic studies.

REFERENCES

1 B. Beermann, Eur J. Clin. Pharmacol , 5 (1972) 28.
2 N. Rietbrock, U. Abshagen, K.V. Bergmann and H. Rennekamp, Eur. J. Clin. Pharmacol., 9 (1975) 105.
3 D. Boerner, A. Olcay, W. Schaumann and W. Weiss, Eur J. Clin. Pharmacol , 9 (1976) 307.
4 N. Rietbrock, J. Guggenmos, J. Kuhlmann and U. Hess, Eur. J. Clin. Pharmacol., 9 (1976) 373.
5 P.H Hinderling, E.R. Garrett and R.C. Wester, J. Pharm. Sci., 66 (1977) 314.
6 D.J. Greenblatt, D.W. Duhm, J. Koch-Weser and T.W. Smith, N. Engl. J. Med., 289 (1973) 651
7 V H. Flasch, B Asmussen and N. Heinz, Arzneim.-Forsch., 28 (1978) 326.
8 W.G. Kramer and R.H. Reuning, J. Pharm. Sci., 67 (1978) 141.
9 W. Schaumann, K. Koch, Naunyn-Schmiedeberg's Arch. Pharmacol., 286 (1974) 195.
10 P.H. Hinderling, E.R. Garrett and R.C. Wester, J. Pharm. Sci., 66 (1977) 242.
11 P.L Malini, F. Sarti, P.R. Dal Monte, A. Grepioni, S. Boschi and E Ambrosioni, Int. J. Clin. Pharm. Res. II, 1 (1982) 21.

12 R.G. Morris, P.Y. Lagnado, D.R. Lehmann, D.B. Frewin, M.L. Glistak and R.B. Burnet, Ther. Drug Monit., 9 (1987) 208.
13 B.-M. Eriksson, L. Tekenbergs, J.-O. Magnusson and L. Molin, J. Chromatogr., 223 (1981) 401.
14 H.A. Nelson, S.V. Lucas and T.P. Gibson, J. Chromatogr., 163 (1979) 169.
15 M.H. Gault, M. Ahmed, N. Tibbo, L. Longerich and D. Sugden, J. Chromatogr., 182 (1980) 465.
16 P. Jakobsen and S. Waldorff, J. Chromatogr., 382 (1986) 349.