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Note

Determination of digoxin, digoxigenin and dihydrodigoxigenin in urine by extraction, derivatization and high-performance liquid chromatography

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The determination of reduced metabolites of digoxin has recently attracted increasing interest [1—12] as the use of an enteric coated formulation of digoxin can give up to 66% of reduced metabolites in DRP excretors [6] compared with the normal levels of 5—15% [1, 5, 8].

Attempts to carry out direct routine determinations of digoxin metabolites in urine by means of high-performance liquid chromatography (HPLC) with UV detection have been unsuccessful owing to the lack of sensitivity [1, 2, 4, 5, 7, 9, 11—13]. Consequently, normal determinations are carried out with tritiated species or by radioimmunoassay. Part of the reason for the failure of UV detection in the HPLC of digoxin and its metabolites in urine is the low yields in the liquid extraction and the fact that it is often necessary to determine as many of the metabolites present as possible.

We describe here another approach, namely the determination of the total hydrolysable amount of reduced and non-reduced species as their aglycones (digoxigenin and dihydrodigoxigenin). The procedure consists of the following steps: hydrolysis to the aglycone by means of incubation [14, 15], extraction of aglycone from urine (or water) by means of an Extrelut column with

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dichloromethane as eluent, derivatization with 4-nitrobenzoyl chloride (4-NBCl) in pyridine and subsequent normal-phase HPLC separation [16, 17].

EXPERIMENTAL

Materials

4-Nitrobenzoyl chloride (analytical-reagent grade; Merck, Darmstadt, F.R.G.) was recrystallized once from *n*-hexane and dried in vacuo. Pyridine was distilled and stored over sodium hydroxide [16, 17]. Digitalis glycosides and aglycones (dihydrodigoxigenin was a mixture of *R* and *S* forms) were purchased from Serva (Heidelberg, F.R.G.). HPLC solvents were of LiChrosolv grade (Merck). All other reagents were of analytical-reagent grade. Extrelut columns were commercial 20-cm columns from Merck.

Instruments

The HPLC equipment was a Spectra-Physics Model 8700 apparatus equipped with an SP 4270 integrator and a Pye Unicam PU 4020 UV detector. A Hibar column (Merck) LiChrosorb Si 60 (5 μm), 20 cm × 4 mm I.D., was used.

Hydrolysis procedure

All glassware was thoroughly cleaned and dried prior to use. To 10 ml of an aqueous solution or urine containing digoxin or aglycone was added 1.00 ml (2.00 ml for urine) of 1.00 *M* hydrochloric acid and the pH was checked (1—2). The solution was incubated at 37°C for 2 h (3 h for urine), 5.0 ml of phosphate buffer (pH 6.5) were added and subsequently 1.00 ml (2.00 ml for urine) of 1.00 *M* sodium hydroxide solution and the pH was checked (6.5—7.0).

Extraction procedure

The hydrolysis mixture was applied to the top of an Extrelut column, the hydrolysis flask was rinsed carefully with a total of 3 ml of water and the washings were applied to the column. After drying for 15 min, the column was eluted with 40 ml of dichloromethane, resulting in ca. 25 ml of eluate. This was evaporated almost to dryness with a stream of nitrogen, the residue was transferred into a 10-ml test-tube with a small volume of dichloromethane, evaporated completely to dryness and further dried in vacuo over concentrated sulphuric acid.

Derivatization

The derivatization solution (100 mg of 4-NBCl in 1 ml of dry pyridine) was prepared by gentle heating. The solution should be used immediately after the preparation.

The residue from the extraction procedure is dissolved in 30 μl of dry pyridine, 20 μl of a standard solution of digitoxigenin in pyridine (2 mg/ml) are added, followed by 150 μl (300 μl for urine extracts) 4-NBCl solution and the mixture is well shaken. The test-tube is stoppered and heated at 70°C for 1 h on a sand-bath, then 2 ml of 5% sodium hydrogen carbonate solution are added and the solution is shaken until the precipitate has dissolved. Chloroform (2.00 ml) is added, the tube is shaken and centrifuged and the

aqueous layer is discarded. The extraction is repeated twice and then three times with 2 ml of 1 *M* hydrochloric acid. The remaining chloroform solution is used directly for HPLC detection.

High-performance liquid chromatography

A 20-μl volume of the chloroform solution was injected through a Rheodyne 100-μl sample loop and eluted with *n*-hexane–dichloromethane–methanol (82.9:14.2:2.9). The flow-rate was 1.2 ml/min at ambient temperature and UV detection at 258 nm was used.

RESULTS AND DISCUSSION

Three series of experiments were carried out. The first consisted in direct derivatization of digoxin, digoxigenin and dihydrodigoxigenin (*R* and *S*), or a mixture thereof, at different concentrations. In the second series the compounds were extracted from water both with and without acid hydrolysis. The third series consisted in extraction of the compounds from urine, using the full procedure described. Commercial compounds were dissolved in drug-free urine.

By comparison of the results from the different series, it was possible to find the extraction recovery (Table I) and the efficiency of the hydrolysis of digoxin.

In the urine experiments, greater amounts of acid for the hydrolysis and derivatization mixture were used than for the aqueous solutions in order to

TABLE I

EFFICIENCY OF EXTRACTION OF DIHYDRODIGOXIGENIN (*R* AND *S*), DIGOXIGENIN AND DIGOXIN

The extraction was performed by the combined hydrolysis–Extrelut extraction–derivatization procedure described. Standard deviations in parentheses (n = 3–8).

Compound	Concentration (μg/ml)	Water (%)	Urine (%)
Dihydrodigoxigenin	10	83.7 (10.9)	74.9 (6.5)
	5	74.3 (10.8)	65.0 (12.0)
	1		79.3 (5.2)
	0		89.3 (6.8)
Digoxigenin*	10	86.7 (9.3)	87.0 (12.3)
	5	90.5 (2.0)	73.2 (8.8)
	1		89.5 (7.7)
Digoxin**	10	93.5 (3.8)	90.2 (4.3)
	6		69.5 (2.3)
	4		96.9 (14.5)
	1		93.0 (7.6)

*At lower concentrations the analysis of urine was found to be unreliable owing to peak coincidence with a urine peak.

**Calculated from peak-height ratios for the digoxigenin peak compared with that for directly derivatized digoxin. The differences in molar absorptivities and molecular weights were taken into account.

ensure hydrolysis and derivatization. The peak-height ratio (compound to digitoxin internal standard) was used throughout the investigation in the analysis of the chromatograms. Digoxin was retained on the Extrelut column, which is why the method requires full hydrolysis of digoxin.

The results show, in accordance with kinetic investigations on the acid hydrolysis of digoxin [14, 15], that the hydrolysis is complete after 2—3 h at 37°C and pH 1—2. Hydrolysis for longer times was attempted but did not change the peak-height ratios.

Dihydrodigoxigenin exists in two enantiomeric forms [10]; Reuning and co-workers [1, 2] recently reported the HPLC separation of the two isomers as their 3,5-dinitrobenzoyl derivatives, but several investigators have reported that

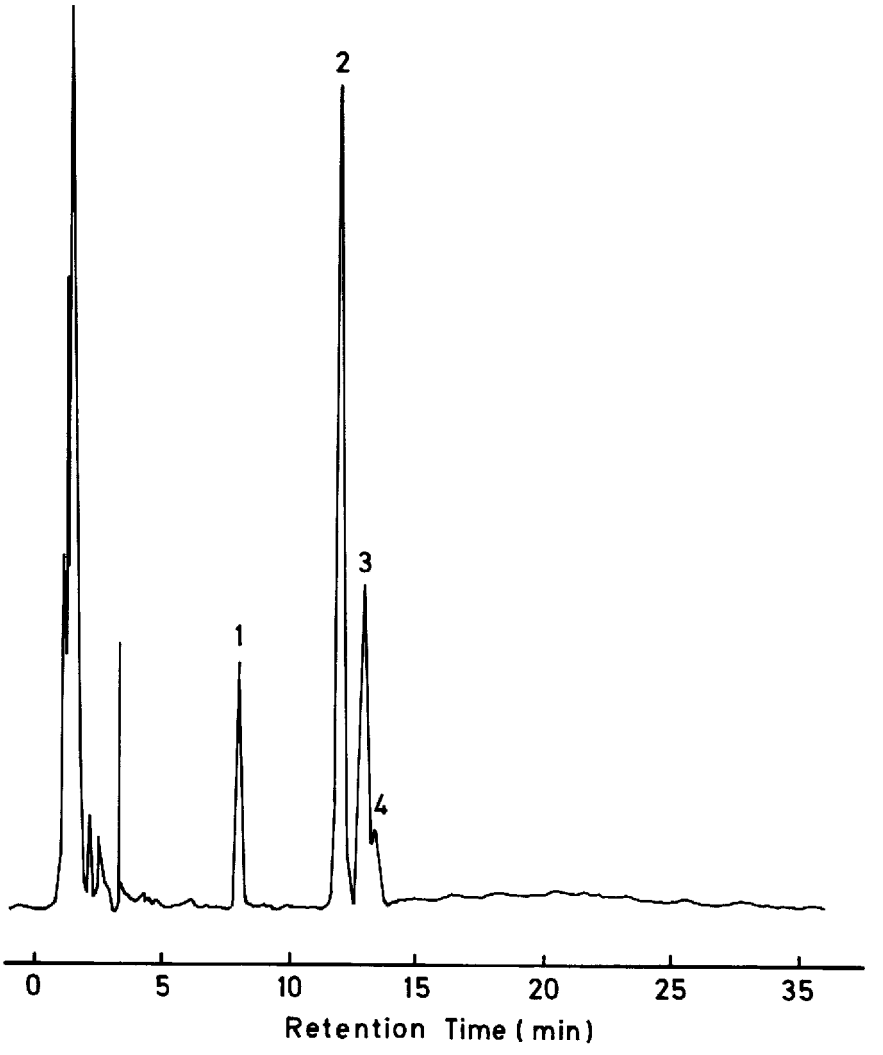

Fig. 1. Representative chromatogram of 4-NBCl derivatives of (1) digitoxigenin standard (40 μg), (2) digoxigenin (100 μg), (3) (*R*)-dihydrodigoxigenin and (4) (*S*)-dihydrodigoxigenin (*R*+*S*, 50 μg). Samples were derivatized directly in pyridine solution. The retention time for digoxin derivatized directly was 44 min.

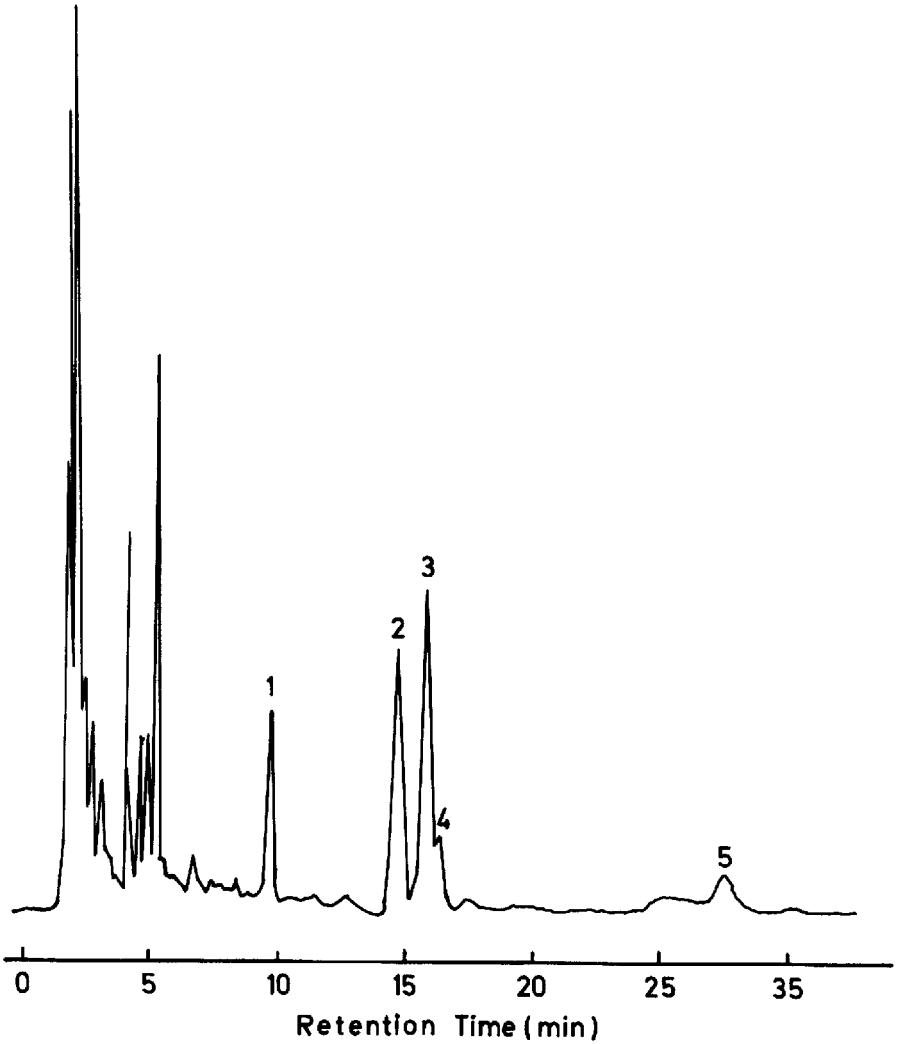

Fig. 2. Chromatogram obtained from a mixture of digoxigenin (50 μg) and (*R*)- and (*S*)-dihydrodigoxigenin (*R*+*S*, 100 μg) extracted from urine following the described method. Peaks: 1 = digitoxigenin standard (40 μg); 2 = digoxigenin; 3 = (*R*)-dihydrodigoxigenin; 4 = (*S*)-dihydrodigoxigenin and urine peak, 5 = urine peak. Chromatograms obtained following hydrolysis—extraction for digoxin and dihydrodigoxigenin gave the same pattern, the "digoxin peak" appearing as its digoxigenin hydrolysate (peak 2).

the reduced compounds are chromatographed with the non-reduced compounds [3, 4, 9, 12, 13].

We found that with the chromatographic system employed the 4-NBCl derivatives of digoxigenin, digoxin and (*R*)- and (*S*)-dihydrodigoxigenin were well separated, as can be seen from Figs. 1 and 2. The two reduced enantiomers could be better separated by changing the eluent mixture, but it was not found necessary in this investigation, as it was recently reported [1] that only the *R* isomer is formed in vivo. The stability of the 4-NBCl derivatives of digoxin and digoxigenin and the derivatization procedure and spectrophotometric

characterisation have been reported by Nachtmann et al. [16, 17]. They found that in their chromatographic system the reduced metabolite could not be separated from the non-reduced compound, whereas our investigations clearly show that it is possible to detect dihydrodigoxigenin as the 4-NBCl derivative.

The reported procedure involves a simpler and more efficient extraction than previously reported. Further, the use of hydrolysis in the first step gives a very simple chromatogram with only two peaks of interest, namely digoxigenin and (*R*)-dihydrodigoxigenin, when applied to human metabolites where only the total amount of reduced versus non-reduced digoxin is of interest.

The UV detection procedure was found not to be sensitive enough for use in routine investigations of human urine species, as found from analyses on urine from two patients undergoing multiple medication. The hydrolysis—extraction procedure reported may, however, be useful with other more sensitive detection methods, giving reasonable results in routine analyses.

REFERENCES

1 R.H. Reuning, T.A. Shepard, B.E. Morrison and H.N. Bockbrader, Drug Metab. Dispos., 13 (1985) 51.
2 H.N. Bockbrader and R.H. Reuning, J. Chromatogr., 310 (1984) 85.
3 B.M. Eriksson, L. Tekenbergs, J.O. Magnusson and L. Molin, J. Chromatogr., 223 (1981) 401.
4 M.H. Gault, L.L. Lougerich, J.C.K. Loo, P.T.H. Ko, A. Fine, S.C. Vasder and M.A. Dawe, Clin. Pharmacol. Ther., 35 (1984) 74.
5 D.R. Clark and S.M. Kalman, Drug Metab. Dispos., 2 (1974) 148.
6 J.O. Magnusson, B. Bergdahl, C. Bogentoft and U.E. Jonsson, Br. J. Clin. Pharmacol., 14 (1982) 284.
7 E. Watson, D.R. Clark and S.M. Kalman, J. Pharmacol. Exp. Ther., 184 (1973) 424.
8 J.O. Magnusson, B. Bergdahl, C. Bogentoft, S. Gustafsson and U.E. Jonsson, Eur. J. Clin. Pharmacol., 27 (1984) 197.
9 H. Greenwood and W. Smedden, Mass Spectrometry in Drug Metabolism, Plenum Press, New York, 1977, p. 179.
10 A. Mostad, Acta Chem. Scand., Ser. B, 36 (1982) 635.
11 J. Lindenbaum, D. Tse-Eng, V.P. Butter and D.G. Rund, Am. J. Med., 71 (1981) 67.
12 M.H. Gault, M. Ahmed, W. Tibbo, L. Longerich and D. Sugden, J. Chromatogr., 182 (1980) 465.
13 H. Gault, J. Kaire, L. Longerich and M. Dawe, Clin. Pharmacol. Ther., 31 (1982) 695.
14 L.A. Sternson and R.D. Shaffer, J Pharm. Sci., 67 (1978) 327.
15 M.H. Gault, J.D. Charles, D.L. Sugden and D.C. Kepkay, J. Pharm. Pharmacol., 29 (1977) 27.
16 F. Nachtmann, H. Spitzy and R.W. Frei, Anal. Chem., 48 (1976) 1576.
17 F. Nachtmann, H. Spitzy and R.W. Frei, J. Chromatogr., 122 (1976) 293.