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DETECTION OF DIGOXIN, DIGITOXIN, THEIR CARDIOACTIVE METABOLITES AND DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—RADIOIMMUNOASSAY

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SUMMARY

High-performance liquid chromatographic (HPLC) systems are described for the separation of the cardioactive metabolites of the digoxin and digitoxin series that are formed by the splitting of digitoxose sugar residues from the aglycone steroid: (1) isocratic separation of digoxin and its cardioactive metabolites; (2) isocratic separation of digitoxin and its cardioactive metabolites; and (3) gradient elution separation of the digoxin and digitoxin series including β-acetyl- and β-methyldigoxin. Separations were performed on a 10-μm bonded octadecyl phase column using various mixtures of acetonitrile—water and acetonitrile—methanol—water as the mobile phase. These methods provide high peak resolution and are well suited for collecting elution fractions, e.g. to link up with sensitive immunological measurements. An HPLC—radioimmunoassay method is described for the quantitation of digoxin, digitoxin and their metabolites in human tissues.

INTRODUCTION

Although the therapeutic effects of cardiac glycosides have long been made use of, the precise and large-scale determination of their concentrations in serum for rational guidance and monitoring of therapy has become possible only since the application of radioimmunoassay (RIA) [1, 2]. Determinations that had to be carried out in the nanogram range showed that other methods such as paper [3, 4], thin-layer [5—8], column [9, 10] and gas—liquid chromatography [11] are insensitive or complicated.

High-performance liquid chromatography (HPLC) has been repeatedly applied and has proved to be a selective, rapid and reproducible method for the determination of digitals glycosides [12—16]. Nevertheless, the minimum detectable amounts are not lower than ca. 10 ng [12, 13] at a signal-to-noise

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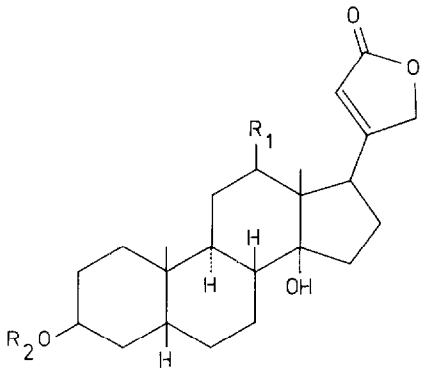

Compound	R_1	R_2
Digitoxigenin (DT-0)	H	H
Digitoxigenin monodigitoxoside (DT-1)	H	D
Digitoxigenin bisdigitoxoside (DT-2)	H	D–D
Digitoxin (DT-3)	H	D–D–D
Digoxigenin (DG-0)	OH	H
Digoxigenin monodigitoxoside (DG-1)	OH	D
Digoxigenin bisdigitoxoside (DG-2)	OH	D–D
Digoxin (DG-3)	OH	D–D–D
β-Acetyldigoxin (β-Ac-DG)	OH	D–D–D–Ac
β-Metnyldigoxin (β-Me-DG)	OH	D–D–D–Me

Fig. 1. Structures of the digitalis glycosides and aglycones investigated. D = Digitoxose; Ac = acetyl; Me = methyl.

ratio of 2:1 as defined in the literature [17]. Fujii et al. [18] achieved an improvement in sensitivity (1 ng for a 0.1-μl injection) by further decreasing the column diameter and the dimensions of the tubing and detector cell. The above studies were concerned with separations of mixtures of pure substances. In biological media, however, the practical detection limit is higher, as in many chromatographic methods [19, 20].

Although HPLC cannot replace immunological methods such as RIA and enzyme immunoassay (EIA) with regard to sensitivity, it is superior in the separation of digitalis metabolites that often cannot be differentiated by immunological means because of considerable cross-reaction with the antibody.

The substances investigated in this study were cardioactive metabolites of digoxin and digitoxin, formed by the successive cleavage of the digitoxose sugar residues from the aglycone steroid. In addition, the clinically important semi-synthetic digoxin derivatives β-acetyldigoxin and β-methyldigoxin were included (Fig. 1). We found all substances of the digoxin and digitoxin series in a study concerning their tissue distribution in man [21] with a high affinity to the RIA antibody between 64 and 160%. For this reason, separations of the cardioactive metabolites by HPLC were developed that allowed quantitation by RIA in the elution fractions collected from the column. The advantageous combination of HPLC and RIA has already been described by other workers [14, 15, 22]. However, up to now no procedure has been demonstrated that makes the combined separation of all the mentioned cardioactive metabolites including β-acetyl- and β-methyldigoxin possible and is further suitable for preparative work. The preparative isolation of all substances allow-

ing subsequent precise quantitation by RIA creates more demands than a pure analytical system.

We therefore established the following demands for suitable HPLC systems: (1) all metabolites should be separated in a single chromatogram; (2) the resolution of two peaks should be high enough (>1.5) to obtain sufficient peak distances when elution fractions are collected; (3) the total elution time should not exceed 30 min; and (4) the mobile phase should be volatile and free from residues.

CHROMATOGRAPHY

Materials

Pumping system. A Series 3B liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) and a dual-pump solvent delivery system suitable for isocratic and gradient operation were used. The mixing ratio of the solvents can be programmed in linear and exponential modes for four intervals (Fig. 2).

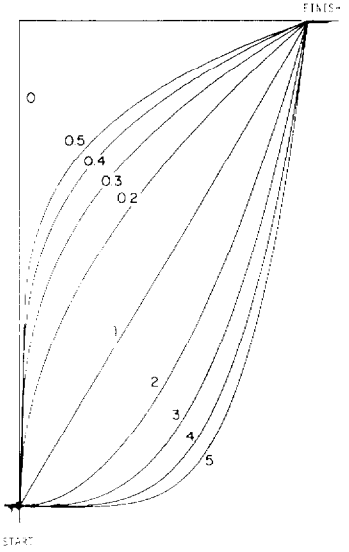

Fig. 2. Gradient curves of the solvent programme. Reproduced from the Handbook of the Series 3 Liquid Chromatograph with permission of Perkin-Elmer.

Samples were introduced on to the column through a Rheodyne 7105 175-μl injection loop (Rheodyne, Berkeley, CA, U.S.A.).

Detector. A Perkin-Elmer LC-85 B dual-beam, variable-wavelength (190—600 nm) detector, equipped with a 2.4-μl flow cell, was used.

Recorder. This was a single-channel compensation writer (Knauer, Berlin, F.R.G.).

Reversed-phase columns. Two Nucleosil C_{18} columns, 25 × 0.40 cm I.D., 7 and 10 μm particle size (Macherey-Nagel, Düren, F.R.G.) were employed.

Reagents. All solvents (water, methanol, acetonitrile) were obtained from Merck (Darmstadt, F.R.G.) and were of HPLC grade. The cardiac glycosides and aglycones listed in Fig. 1 were purchased from Serva (Heidelberg, F.R.G.).

Procedure

Separate stock solutions of each cardenolide were prepared by dissolution in 100% methanol. Complex solutions of the metabolites of the digoxin series, the digitoxin series and of all investigated substances were prepared additionally. Each compound was chromatographed separately to determine its purity and retention time. The injection volumes for the chromatograms shown were 20—40 μl. The flow-rate for the 10-μm column was fixed near its optimal selectivity at 1 ml/min. Degassing of the solvent mixtures was carried out for 15 min in an ultrasonic bath. The pressures in the chromatograms illustrated varied with the solvent composition within the range 65—85 bar. The absorbance detector was set at 220 nm near the absorption maximum of the compounds (butenolide ring). A calibration graph was constructed for digoxin, digitoxin and their aglycones using the average peak areas from three chromatograms.

Results and Discussion

By increasing the portion of water in a mobile phase containing acetonitrile or methanol, the eluting power of the solvent could be varied over a wide range. With a binary isocratic solvent system the separation of the metabolites of the digoxin series (DG-0, DG-1, DG-2 and DG-3, Fig. 3) and of the digitoxin series (DT-0, DT-1, DT-2 and DT-3) was not completely satisfactory. With acetonitrile—water (20:80) we achieved a good enough resolution of the digoxin series for preparative work. However, under these conditions the capacity factors exceeded considerably the values between 1 and 5 favourable for isocratic systems. Improving the last parameter by increasing the eluting power of the mobile phase (acetonitrile—water, 25:75) led in particular to a poorer discrimination of the metabolites DG-0 and DG-1, so that a baseline resolution was no longer possible. For the digitoxin series we encountered the

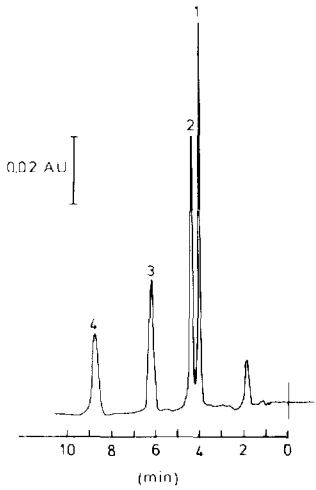

Fig. 3. Isocratic separation of digoxin and its metabolites. Conditions: column, Nucleosil C_{18}, 10 μm; mobile phase, acetonitrile—water (25:75); flow-rate, 1 ml/min; UV monitor at 220 nm; 1 μg of each compound injected in 20 μl of methanol. Peaks: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin.

same problem at a lower polarity of the mobile phase (acetonitrile—water, 40:60). By using the 7-μm Nucleosil C_{18} column we could not significantly improve the results. The peak resolution in these systems is more dependent on the injection volumes. For the uniform dissolution of all cardenolides, pure methanol was most suitable. As the test solutions are more eluotropic than the mobile phase in this instance, the peak width increased with increasing injection volume. However, sample volumes between 20 and 40 μl used to obtain the chromatograms illustrated showed no negative influence.

With a ternary solvent system we achieved a significant improvement in the isocratic mode. Although acetonitrile and methanol do not essentially differ in their polarity, other solvent parameters differ according to whether they are a hydrogen donor or acceptor. We found a favourable change in the solvent properties of the mobile phase by combining acetonitrile, methanol and water. With this mixture good separations were accomplished for the metabolites of both the digoxin and digitoxin series (Figs. 4 and 5, Table I). Digoxin and its cardioactive metabolites DG-2, DG-1 and DG-0 were separated with high resolution ($R \geqslant 3.0$) in 13 min using acetonitrile—methanol—water (20:20:60).

The simultaneous separation of the digitoxin series was not possible under these conditions, however. The elution of the DT series made a lower polarity of the mobile phase necessary. Increasing the proportion of acetonitrile in the solvent system for this purpose had a greater effect on retention times than a corresponding increase in the proportion of methanol. When the eluotropic

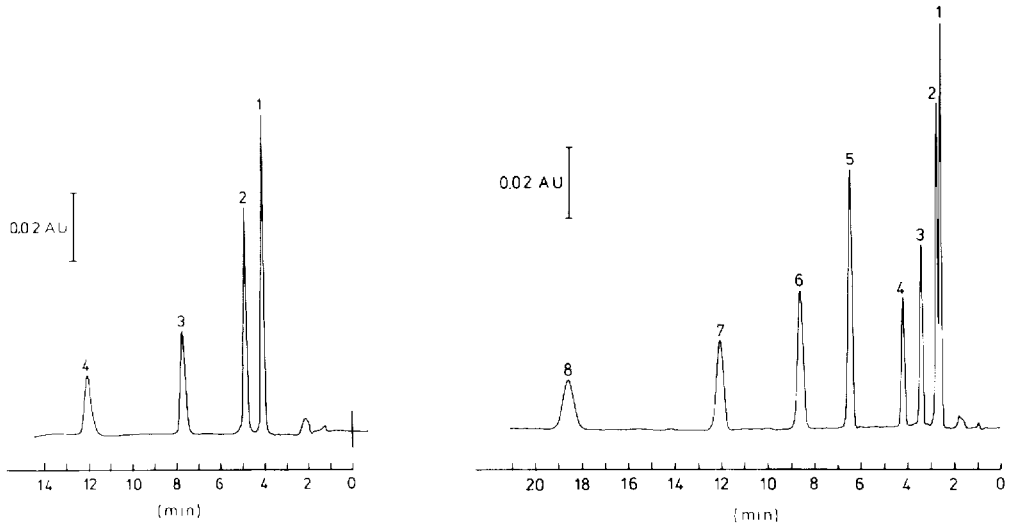

Fig. 4. Isocratic separation of digoxin and its metabolites. Conditions: column, Nucleosil C_{18}, 10 μm; mobile phase, acetonitrile—methanol—water (20:20:60); flow-rate, 1 ml/min; UV monitor at 220 nm; 1 μg of each compound injected in 20 μl of methanol. Peaks: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin.

Fig. 5. Isocratic separation of the digoxin and digitoxin series of glycosides. Conditions: column, Nucleosil C_{18}, 10 μm; mobile phase, acetonitrile—methanol—water (30:30:40); flow-rate, 1 ml/min; UV monitor at 220 nm; 1 μg of each compound injected in 40 μl of methanol. Peaks: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin; 5 = digitoxigenin; 6 = digitoxigenin monodigitoxoside; 7 = digitoxigenin bisdigitoxoside; 8 = digitoxin.

TABLE I

TOTAL RETENTION TIMES AND RESOLUTION OF SOME CRITICAL PAIRS OF BANDS OF THE CARDENOLIDES IN THE CHROMATOGRAMS DEMONSTRATED

Compound	Retention time (min)			
	Fig. 3	Fig. 4	Fig. 5	Fig. 6
DG-0	4.1	4.1	2.6	6.4
DG-1	4.4	4.9	2.8	8.3
DG-2	6.3	7.7	3.4	10.5
DG-3	8.8	12.0	4.2	11.5
β-Methyldigoxin				15.7
β-Acetyldigoxin				17.3
DT-0			6.5	18.9
DT-1			8.6	20.1
DT-2			12.1	22.6
DT-3			18.6	24.1
	Resolution			
DG-0/DG-1	1.2	3.0	1.0	4.2
DT-0/DT-1			4.8	2.1

strength of the solvent was kept constant, an increase in the percentage of methanol led to a higher selectivity of the chromatographic system for the metabolites in the digoxin and digitoxin series. The separations of DG-0/DG-1 and DT-0/DT-1 were particularly improved. The resolution of the metabolites of the DT series proved to be unproblematic and high ($R \geqslant 4.8$) with acetonitrile—methanol—water (30:30:40). The great difference in the polarities of the substances investigated did not allow the common separation of all cardenolides, sufficient for quantitative preparative work, in the isocratic mode. The chromatogram demonstrated in Fig. 5 just allows the differentiation of DG-0 and DG-1 for analytical purposes.

The problems described here made gradient elution seem promising. The apparatus used worked with two electronically controlled pumps as a high-pressure gradient system. The more polar, aqueous organic solvent was forced by pump B. Corresponding to the graph in Fig. 2, pure organic solvent was added by pump A. The final mixing ratio could be programmed in four successive intervals (T_1—T_4). At the end of a chromatographic run the column was washed with pure solvent if necessary (T_{purge}). Before starting a new run the column was re-equilibrated for 10 min. As for the isocratic mode, the flow-rate was fixed at 1 ml/min. Gradient elution separations were developed for methanol—water and acetonitrile—water as eluents. If the shift in the baseline is not automatically compensated for by the recorder, acetonitrile—water should be used because of its better UV transparency at the detection wavelength of 220 nm.

Fig. 6 shows a gradient elution separation of all cardenolides investigated in a single chromatogram. The programme of the complex (linear, exponential) gradient elution is given in Table II. In addition to the resolution of the cardioactive metabolites of the DG and DT series, we demonstrated the simul-

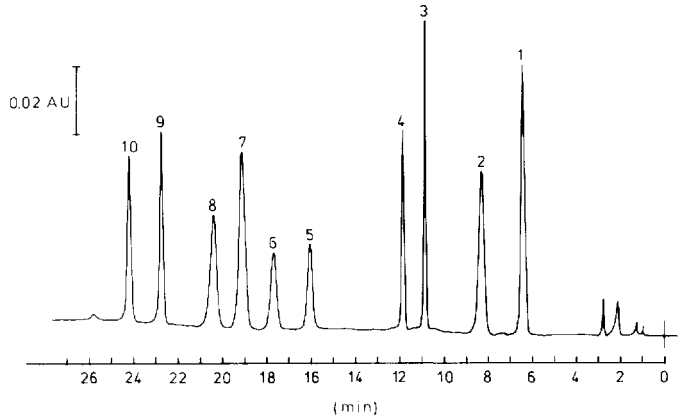

Fig. 6. Separation of digoxin, digitoxin, their cardioactive metabolites and semi-synthetic derivatives by a complex gradient elution. Conditions: column, Nucleosil C_{18}, 10 μm; mobile phase, solvent system at time 0: acetonitrile—water (20:80, w/w); solvent was changed according to the gradient profile in Table II; flow-rate, 1 ml/min; UV monitor at 220 nm; 1 μg of each compound injected in 40 μl of methanol. Peaks: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin; 5 = β-methyldigoxin; 6 = β-acetyldigoxin; 7 = digitoxigenin; 8 = digitoxigenin monodigitoxoside; 9 = digitoxigenin bisdigitoxoside; 10 = digitoxin.

TABLE II

HPLC SOLVENT PROGRAMME

Complex gradient elution constructed from four gradient segments. The gradient slope (curves 0.3, 1, 4, respectively) of each interval (T_1—T_4) can be drawn from Fig. 2.

Column: Nucleosil C_{18}, 10 μm
Solvents: pump A: acetonitrile
pump B: acetonitrile—water (20:80, w/w)

UV monitor: 220 nm
Flow-rate: 1 ml/min
Percentage A: 0.1
Pressure: ca. 80 bar

T_1: 3 min
Curve: 1
Percentage A: 0.1 (v/v)

T_2: 1 min
Curve: 1
Percentage A: 15 (v/v)

T_3: 12 min
Curve: 4
Percentage A: 20 (v/v)

T_4: 12 min
Curve: 0.3
Percentage A: 40 (v/v)

$T_{equil.}$: 10 min
Percentage A: 0.1 (v/v)

taneous separation of the clinically important digoxin derivatives β-acetyl- and β-methyldigoxin. They elute between DG-3 and DT-0.

The resolution of all peaks is remarkably high, with $R \geqslant 2.1$, and thus allows easy preparative work. The peak symmetry proved to be good ($T \leqslant 1.3$). The repeatability of the retention times for each compound in the gradient system was determined. The relative standard error was less than 1.5% in all

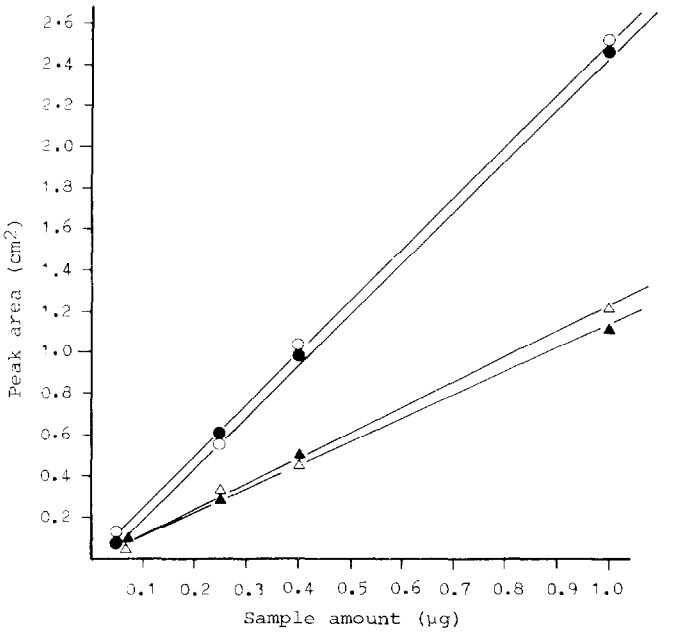

Fig. 7. Calibration graphs for digoxin (△), digoxigenin (○), digitoxin (▲) and digitoxigenin (●).

instances (n = 6). Calibration graphs were constructed for digoxin, digitoxin and their aglycones at an attenuation of 0.16 a.u.f.s. using the gradient system (Fig. 7). The graphs for the monodigitoxosides and bisdigitoxosides have intermediate slopes. This behaviour is due to the fact that the UV absorbance is derived from the unsaturated lactone ring of the steroid nucleus and remains unaffected by the digitoxose sugars, which only add to the molecular weight.

As the elution volumes of all compounds are fairly constant in the gradient mode, quantitation can be carried out with high sensitivity by the peak height method. The detection limit of the last eluting substance, digitoxin, at a signal-to-noise ratio of 3:1 was 10 ng.

DETERMINATION OF DIGOXIN, DIGITOXIN AND THEIR CARDIOACTIVE METABOLITES IN HUMAN TISSUES

Materials

Extraction columns. These were Clin Elut (Analytichem International, Harbor City, CA, U.S.A.), for sample preparation by liquid—liquid extraction, designed to take up 20 ml of aqueous phase.

Ultrasonic pulse instrument. A Labsonic (Braun, Melsungen, F.R.G.) was used.

Digoxin radioimmunoassay. RIA phase™ (Beckman, Fullerton, CA, U.S.A.), a ^{125}I solid-phase second antibody RIA was employed.

Digitoxin radioimmunoassay. Digitoxin RIA kit (Becton Dickinson, New York, NY, U.S.A.), a ^{125}I RIA was utilized.

Reagents. Solvents (ethanol, methanol, light petroleum, ethyl acetate, water) were obtained from Merck and were of analytical-reagent grade.

Samples. Autopsy samples from patients being treated with therapeutic doses of β-acetyldigoxin or β-methyldigoxin, from a group of patients on maintenance treatment with digitoxin and from a case of fatal digoxin intoxication, were investigated. Tissue specimens were taken from the myocardium of the left ventricle and from the kidney comprising approximately equal parts of cortex and medulla. Data on the quantitative distribution of the metabolites mentioned (Fig. 1) should be obtained [21].

Tissue extraction

Tissue specimens weighing 1 g were taken at autopsy and stored in closed vials under refrigeration. At the time of determination adherent fat was cut off with scissors. The samples were transferred into a laboratory tissue grinder and homogenized in 20 ml of 70% ethanol. The homogenate was exposed (with cooling) to an ultrasonic probe (3 cm^2), delivering an energy of 35 $J/cm^2 \cdot s$ for 15 min. After magnetic stirring for further 12 h the mixture was centrifuged and the supernatant collected. The alcoholic phase was transferred into a separating funnel, 10 ml of light petroleum were added and the mixture was shaken vigorously by hand. The upper phase was discarded, 4 ml of water were added and the extraction step was repeated once. The alcoholic phase was placed in a round-bottomed flask and evaporated to dryness on a rotary evaporator. The residue was dissolved in 20 ml of distilled water and the aqueous sample was placed on to a Clin Elut column. After distribution of the phase on the column matrix, 60 ml of ethyl acetate were added and the extraction solvent was collected in a cup placed under the outlet. The eluate was evaporated to dryness and the residue redissolved in 40 μl of methanol.

Recovery

The recovery of the extraction was determined by addition of [^{3}H]digoxin and [^{3}H]digitoxin to the homogenate before extraction. The recovery was 92% for [^{3}H]digoxin and 97% for [^{3}H]digitoxin. The recovery of all cardenolides investigated (Fig. 1) was measured by HPLC. The extraction procedure was carried out with pure substances, leaving any tissue material. A 1-μg amount of each cardenolide was added to the 70% ethanol phase. Recoveries were between 63 and 89% (except for β-acetyldigoxin, 40%). In a radio-chromatogram, obtained with [^{3}H]digoxin, no degradation products could be detected throughout the extraction procedure.

HPLC—RIA quantitation

The sample residue from the Clin Elut column, reconstituted in 40 μl of methanol, was injected together with 100 ng of propyphenazon as an internal standard into the injection port of the chromatograph. Using an HPLC gradient separation (Fig. 6) all metabolites were separated. With the extraction and purification technique used, the tissue extracts could be freed from impurities to the extent that no overloading effects were observed. Elution fractions were collected according to the retention times and evaporated to dryness by lyophilization. RIA quantitation was carried out after dissolving the residue in 1 ml of blank serum. A commercial digoxin RIA (Beckman) and digitoxin

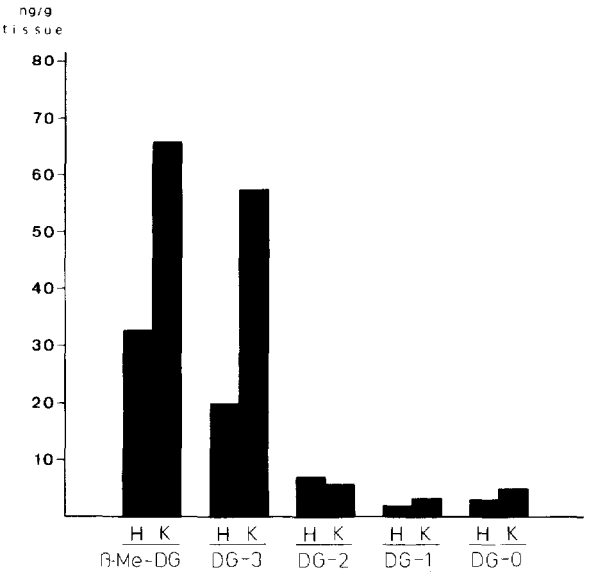

Fig. 8. Distribution of cardioactive metabolites in post mortem tissue samples of a patient after long-term treatment with therapeutic doses of β-methyldigoxin (0.2 mg per day). H = myocardium (left ventricle); K = kidney. β-Me-DG = β-methyldigoxin; DG-3 = digoxin; DG-2 = digoxigenin bisdigitoxoside; DG-1 = digoxigenin monodigitoxoside; DG-0 = digoxigenin.

RIA (Becton Dickinson) were used. For the assay of each cardioactive metabolite its own calibration graph was constructed. The affinities of the cardioactive metabolites of the digoxin series to the digoxin antibody were measured to fall between 64 and 119% and for the digitoxin series we found values between 100 and 160%, respectively. In this way all cardioactive metabolites could be assayed without a decrease in sensitivity. Fig. 8 demonstrates a typical metabolic pattern determined by the HPLC—RIA method.

CONCLUSION

The HPLC systems reported in this paper provide isocratic programme modes for the separation of the cardioactive metabolites of the digoxin and digitoxin series with high resolution. The simultaneous separation of both series of glycosides, including the clinically relevant derivatives β-acetyl- and β-methyldigoxin, was achieved by a complex gradient elution. As the peak resolution is generally high and the elution volumes of all cardenolides are fairly constant, the gradient system is suitable for collecting elution fractions. Hence HPLC appears to be an ideal method for the rapid separation and isolation of complex mixtures of digitalis metabolites that cannot be differentiated by immunological means. A subsequent, highly sensitive quantitation can be carried out by RIA after having evaporated the HPLC fractions to dryness and dissolving the residue in buffer or blank plasma. We applied this technique successfully to the determination of cardioactive digoxin and digitoxin metabolites in human heart and kidney [21]. This represents a favourable addition to immunological methods such as RIA and EIA. By preliminary HPLC many

substances inherent in biological media that potentially cross-react with the antibody are excluded. The specificity and reliability of the measurement of individual compounds can thus be improved.

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