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RAPID AND SENSITIVE HIGH-RESOLUTION PROCEDURE FOR DIGITALIS GLYCOSIDE ANALYSIS BY DERIVATIZATION LIQUID CHROMATOGRAPHY

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SUMMARY

The separation and quantitative determination of digitalis glycosides by high-performance liquid chromatography following derivatization with 4-nitrobenzoylchloride (4-NBCl) is described. The compounds of primary interest were the digitalis glycosides and aglycones of the pharmaceutically important A, B and C series. The derivatization step results in higher extinction values at a more favourable wavelength (260 nm), which permits the use of low-cost ultraviolet detectors. Detection limits are below 20 ng/ml for all of the glycosides tested. The chromatographic properties are also improved by reducing the polarity without a decrease in selectivity. The use of low-polarity and low-viscosity solvent systems on silica gel adsorbents permits rapid isocratic separations of complex mixtures as they usually occur in pharmaceutical products and extracts.

The quantitative potential of this method was demonstrated by analyzing ampoule solutions containing desacetyl lanatoside C as the active compound. The active substance, by-products and degradation products were determined down to 0.1% of the total glycoside concentration in one ampoule.

INTRODUCTION

The determination of low concentrations of digitalis glycosides by high-performance liquid chromatography (HPLC) is to some extent a detection problem as the only ultraviolet (UV) absorption maximum at 220 nm, due to the α,β-unsaturated lactone ring, is relatively weak. The relatively high polarity and the widely differing solubilities also place some restrictions on the chromatographic possibilities[1].

The methods known so far for the determination of digitalis glycosides are usually associated with thin-layer chromatography (TLC) and involve the formation

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of either coloured[2–6] or fluorescing[7–9] products. A review of available methods has been given by Page[10]. Derivatization is required if gas chromatography is to be used and the resulting degradation reactions make a pre-separation mandatory[11,12].

Recently, esterification of these glycosides with 4-nitrobenzoylchloride has been developed to give derivatives with more desirable detection and chromatographic properties[13]. The quantitative derivatization involves all OH groups on the sugar and also the steroid portion, except for the 14-OH group. The glycoside remains intact. The reaction conditions and resulting products have also been investigated[13]. This method opens new possibilities for the analysis of this pharmaceutically interesting group of compounds. It was the purpose of this work to investigate these possibilities and to develop an HPLC method suitable for the quantitation of digitalis glycosides and aglycones in pharmaceutical preparations.

EXPERIMENTAL

Reagents

4-NBCl (analytical grade, Fluka, Buchs, Switzerland), was recrystallized once from light petroleum (b.p. 60–70°, Riedel de Haen, Seelze-Hannover, G.F.R.). The melting point should be between 71 and 73°. Analytical-grade pyridine (Fluka) was refluxed for 3 h with sodium hydroxide (analytical grade, E. Merck, Darmstadt, G.F.R.), distilled and stored over sodium hydroxide (b.p. 115–116°).

4-Dimethylaminopyridine (purum, Fluka) and acetonitrile (Uvasol quality, Merck) were used. All other reagents and solvents were of analytical grade (Merck). The digitalis glycosides and aglycones (see Table I) were provided by Sandoz (Basle, Switzerland).

Merckosorb SI 60 silica gel of average particle size 5 μm was employed for HPLC.

Instruments

The HPLC was carried out on a Hewlett-Packard UFC-1000 chromatograph equipped with a DuPont 842 UV detector (254 nm) and on a Siemens S 200 instrument equipped with a Zeiss PM-4 spectrometric detector (260 nm).

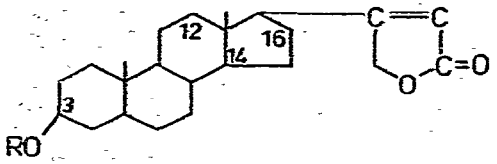
Derivatization

A fresh reagent solution has to be prepared every day. A 100-mg amount of 4-NBCl was dissolved per millilitre of pyridine with gentle warming.

The glycosides were also dissolved in pyridine. The reaction was carried out in stoppered 10-ml centrifuge tubes. To 50 μl of a glycoside solution containing not more than 0.5 mg of the glycoside, 150 μl of reagent solution were added and the mixture was well shaken and allowed to react for 10 min at room temperature. After this period, the reaction was shown to be quantitative by TLC. The centrifuge tubes were embedded in a beaker filled with sand at a temperature of 50°. This sand-bath was placed in a desiccator and the pyridine removed by a water suction vacuum within 10 min. The centrifuge tubes were flushed with a stream of air or nitrogen and 2 ml of 5% sodium carbonate solution which also contained 5 mg of 4-dimethylaminopyridine were added. The excess of reagent was hydrolyzed after 5 min of shaking or treatment in an ultrasonic bath. A blank treated simultaneously should yield a clear

TABLE I

CHEMICAL STRUCTURES OF THE DIGITALIS GLYCOSIDES AND AGLYCONES INVESTIGATED



Compound	*12-*	*14-*	*16-*	*R**
Digitoxigenin	H	OH	H	H
Digitoxigenin monodigitoxoside	H	OH	H	D-
Digitoxigenin bisdigitoxoside	H	OH	H	D-D-
Digitoxin	H	OH	H	D-D-D-
Acetyldigitoxin	H	OH	H	AcD-D-D-
Lanatoside A	H	OH	H	G-AcD-D-D-
Desacetyl lanatoside A	H	OH	H	G-D-D-D-
Gitoxigenin	H	OH	OH	H
Lanatoside B	H	OH	OH	G-AcD-D-D-
Desacetyl lanatoside B	H	OH	OH	G-D-D-D-
Digoxigenin	OH	OH	H	H
Digoxigenin monodigitoxoside	OH	OH	H	D-
Digoxigenin bis-digitoxoside	OH	OH	H	D-D-
Digoxin	OH	OH	H	D-D-D-
Acetyldigoxin	OH	OH	H	AcD-D-D-
Lanatoside C	OH	OH	H	G-AcD-D-D-
Desacetyl lanatoside C	OH	OH	H	G-D-D-D-
Lanatoside D	OH	OH	OH	G-AcD-D-D-
Diginatigenin	OH	OH	OH	H
Diginatin	OH	OH	OH	D-D-D-
Lanatoside E	H	OH	-O-C-H (O)	G-AcD-D-D-
Gitaloxigenin	H	OH	-O-C-H (O)	H
Gitaloxin	H	OH	-O-C-H (O)	D-D-D-

* D = digitoxose; AcD = acetyldigitoxose; G = glucose.

solution (the derivatives are poorly soluble in water and give turbid solutions). The derivatives were then extracted with 2 ml of chloroform and treated with 2 ml of 5% sodium hydrogen carbonate solution and twice with 3 ml of 0.05 *N* hydrochloric acid containing 5% of sodium chloride.

This procedure gives quantitative isolation of the derivatives and complete exclusion of excess of reagent and pyridine.

Extraction of an ampoule solution

To the contents of one ampoule (2 ml) were added 15 ml of 2% sodium hydrogen carbonate solution. The solution was extracted five times with 10 ml of

chloroform–2-propanol (3:2), and each extract was washed with the same 10 ml of water starting with the first extract. This washing procedure was repeated with a further 10 ml of water. The combined fractions were filtered into a 100-ml round-bottomed flask with some of the chloroform–2-propanol solvent mixture. After evaporation to dryness, the residue was transferred into a 10-ml centrifuge tube with two 1-ml volumes of chloroform–pyridine (10:1) and the solvent evaporated at 50° on the sand-bath. The reagent was added directly to this residue. The use of about 200 μl of reagent is recommended for the derivatization.

HPLC of the derivatives

The columns were packed with the Merckosorb SI 60 silica gel by a balanced slurry technique[14]. The columns were 15 or 20 cm long and of 3 mm I.D. The mobile phase consisted of the following three-component mixtures: (1) *n*-hexane–chloroform–methanol; (2) *n*-hexane–chloroform–acetonitrile; and (3) *n*-hexane–methylene chloride–acetonitrile.

The chloroform extract containing the derivatives was injected directly on to the column with a Siemens injector (10 μl), a Valco loop injector (25 μl) or a Rheodyne 7105 universal injection system (10–100 μl). Separations were carried out at ambient temperatures (20–23°), thermostating being unnecessary. The details are given separately with each chromatogram.

RESULTS AND DISCUSSION

Chromatography

Of all the solvent systems tested, the best separations were achieved with the above three-component solvent systems.

Systems containing acetonitrile showed the best selectivity; ether-containing phases were unsuitable.

The k' values of the derivatives of some important digitalis glycosides using solvent system 1 with different proportions of the components are given in Table II. At flow-rates of 1.5–2.0 ml/min the k' values were constant. Chloroform and methanol exhibited different selectivities. The separation of the 4-NB derivatives of gitoxigenin and digoxigenin was improved with an increase in chloroform content. Gitoxigenin was eluted first and overlapped with digitoxigenin using *n*-hexane–chloroform–methanol (10:3:0.5). With an increase in the methanol content, gitoxigenin was shifted in the direction of digoxigenin. Good separations were also achieved between acetyldigitoxin and digitoxin and acetyldigoxin and digoxin.

Solvent system 1 was not suitable for the separation of the lanatosides. A separation of five digitalis components of the digitoxin series as their 4-NB derivatives is shown in Fig. 1. The complete separation takes 11 min.

More complex mixtures can be separated successfully with solvent systems 2 and 3. The k' values of a number of derivatives of glycosides and aglycones are given in Table III. It is possible to separate isocratically the entire polarity range from the aglycones to the lanatosides and desacetyl lanatosides. It is interesting that the order of separation does not necessarily follow the order of polarities. A monosubstituted digitoxigenin, for example, always appears after the corresponding disubstituted

TABLE II

RETENTION DATA FOR SOME DERIVATIZED DIGITALIS GLYCOSIDES FOR SOLVENT SYSTEM 1

Values given are k' values determined from five separate chromatograms. Apparatus: Siemens S 200; detector, Zeiss PM 4, $\lambda = 260$ nm; column, SI 60, 5 μm, 20 cm × 3 mm I.D.; flow-rate, 1.5–2.0 ml/min.

4-NB derivative	*Proportions in n-hexane–chloroform–methanol mixture*			
	10:1:0.5	*10:2:0.5*	*10:3:0.5*	*10:1:1*
Digitoxigenin	4.01	2.68	1.93	2.42
Gitoxigenin	5.63	3.08	1.95	3.19
Digoxigenin	6.79	4.19	2.74	3.48
Digitoxigenin mono-digitoxoside	8.04	3.90	2.41	3.89
Digitoxigenin bis-digitoxoside	14.4	5.77	2.99	5.83
Acetyldigitoxin	18.1	6.59	3.26	6.73
Digitoxin	25.0	8.50	3.85	8.57
Acetyldigoxin	> 25	9.52	4.44	9.38
Digoxin	> 25	12.0	5.16	11.9
Lanatoside A	> 25	23.3	8.17	23.9
Lanatoside B	> 25	> 25	8.61	> 25
Lanatoside C	> 25	> 25	11.4	> 25
Desacetyl lanatoside C	> 25	> 25	> 20	> 25

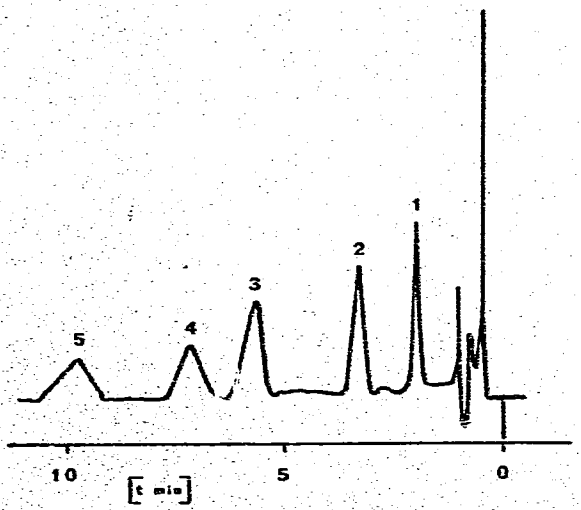


Fig. 1. HPLC of some digitalis glycoside 4-NB derivatives of the digitoxin group. 1 = Digitoxigenin; 2 = digitoxigenin monodigitoxoside; 3 = digitoxigenin bisdigitoxoside; 4 = acetyldigitoxin; 5 = digitoxin. The peaks in front are from impurities of reagents and chloroform. Solvent system: *n*-hexane–chloroform–methanol (10:1:0.5). Column: SI 60, 5 μm, 20 cm × 3 mm I.D. Flow-rate: 1.5 ml/min; $p = 100$ atm. Injection volume: 10 μl. Apparatus: Siemens S 260 with Zeiss PM-4 UV detector set to 260 nm.

TABLE III

RETENTION DATA FOR DERIVATIZED DIGITALIS GLYCOSIDES WITH SOLVENT SYSTEMS 2 AND 3

Values given are k' values determined from five separate chromatograms. Apparatus: Hewlett-Packard UFC 1000; detector, DuPont 842, $\lambda = 254$ nm; column, SI 60, 5 μm, 15 cm × 3 mm I.D.; flow-rate, 1.35–1.4 ml/min.

4-NB derivative	*Proportions in solvent systems*		
	n-Hexane–methylene chloride–acetonitrile		*n-Hexane–chloroform–acetonitrile; 30:10:9 (2)*
	10:3.5:2.5 (3a)	*10:3:3 (3b)*	
Digitoxigenin	4.68	3.87	3.07
Gitoxigenin	3.55	3.09	2.72
Digoxigenin	7.41	5.66	4.77
Diginatigenin	4.42	3.73	3.52
Gitaloxigenin	8.06	6.19	5.01
Digitoxigenin monodigitoxoside	6.55	5.19	4.31
Digitoxigenin bisdigitoxoside	9.17	6.92	5.93
Digoxigenin monodigitoxoside	9.36	6.96	6.40
Digoxigenin bisdigitoxoside	13.0	9.08	8.42
Acetyldigitoxin	11.6	8.13	6.93
Digitoxin	12.9	9.14	8.15
Gitoxin	10.6	7.75	7.51
Acetyldigoxin	16.5	10.7	9.90
Digoxin	18.3	11.9	11.4
Diginatin	12.4	8.77	9.16
Gitaloxin	21.1	13.9	12.9
Lanatoside A	32.7	19.1	18.4
Lanatoside B	27.6	16.0	16.9
Lanatoside C	46.0	24.6	25.6
Lanatoside D	32.1	18.0	20.1
Desacetyl lanatoside A	39.5	22.8	23.1
Desacetyl lanatoside B	32.3	19.3	21.0
Desacetyl lanatoside C	55.5	28.9	32.2

gitoxigenin. With system 3b, even the trisubstituted diginatigenin appears before digitoxigenin.

Using system 2, the order of elution of diginatigenin and digitoxigenin is reversed and the separation is much improved. The selectivity for the acetylated and desacetylated glycosides is also improved with system 2. The selectivity factor for the pair digoxin–acetyldigoxin is $\alpha = 1.11$ with solvent system 3b and $\alpha = 1.15$ with system 2.

The three-component mixtures used for the investigations are nearly saturated systems. This is particularly true for the mixture *n*-hexane–methylene chloride–acetonitrile (10:3:3) (3b) which at about 20° comes very close to the saturation point. It is therefore conceivable that the partition mechanism in these separation processes is similar to those postulated by Huber *et al.*[15] and Hesse and Hövermann[16]. The mixtures discussed here are easier to handle, however.

Fig. 2 shows the separation of seven compounds of the C-series with solvent system 3b. The chromatograms for three compounds each of the A, B, C, D and E series are shown in Fig. 3, obtained using solvent system 2.

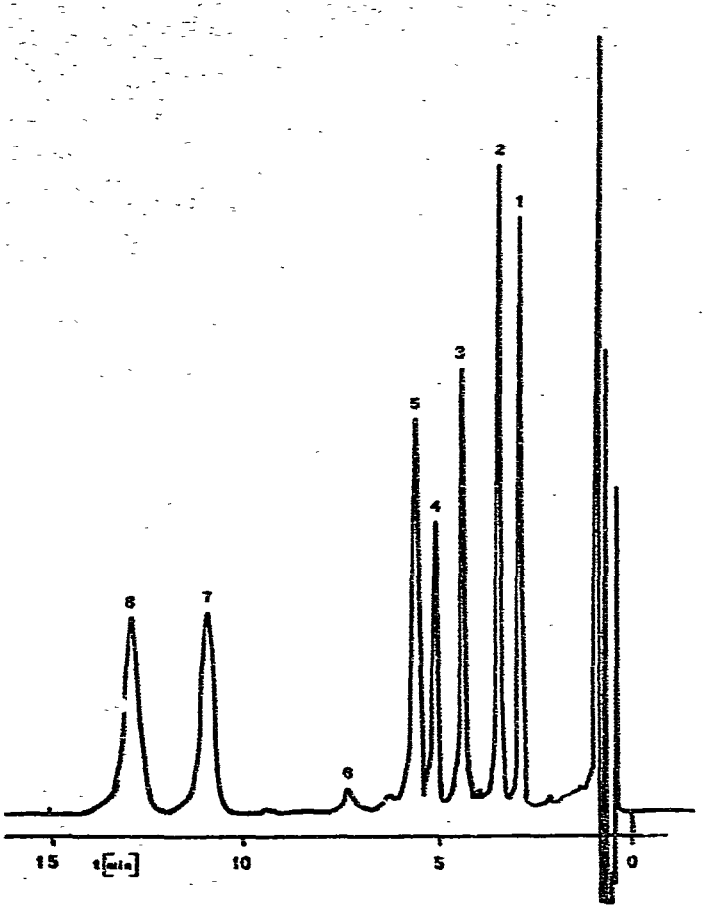


Fig. 2. HPLC of some digitalis glycoside 4-NB derivatives of the C series. 1 = Digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = acetyldigoxin; 5 = digoxin; 6 = unknown by-product of 8; 7 = lanatoside C; 8 = desacetyl lanatoside C. Solvent system: *n*-hexane–methylene chloride–acetonitrile (10:3:3). Column: SI 60, 5 μm, 15 cm × 3 mm I.D. Flow-rate: 1.5 ml/min; p = 120 atm. Apparatus: Hewlett-Packard UFC 1000 equipped with a Du-Pont 842 UV detector (λ = 254 nm). Injection: Rheodyne 7105 injection system; 20-μl injection volume in chloroform.

The selectivity of the 4-NB derivatives of similar glycosides is improved considerably with an increase in the size of the sugar moiety (higher degree of substitution). A typical example is the separation of the pair gitaloxin–digoxin in comparison with the pair gitaloxigenin–digoxigenin. The only exception is the pair gitoxin–digitoxin.

By studying other combinations of the solvent systems one can find the required selectivity for any glycoside combination. An example is given in Fig. 4. The five aglycones digitoxigenin, gitoxigenin, digoxigenin, gitalcxigenin and diginatigenin were separated with the four-component mixture *n*-hexane–methylene chloride–acetonitrile–methanol 35:6:2.5:1. One can see that the order of elution has changed in comparison with solvent system 2.

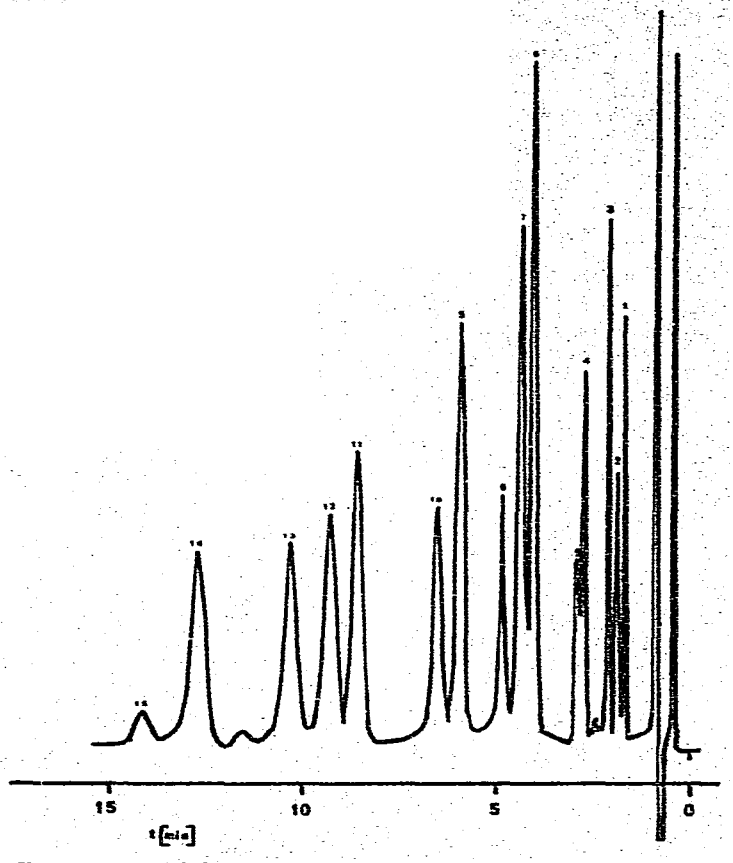


Fig. 3. HPLC of three 4-NB derivatives each of the A, B, C, D and E series of digitalis glycosides. 1 = Gitoxigenin; 2 = digitoxigenin; 3 = diginatigenin; 4 = digoxigenin; 5 = gitaloxigenin; 6 = gitoxin; 7 = digitoxin; 8 = diginatin; 9 = digoxin; 10 = gitaloxin; 11 = lanatoside B; 12 = lanatoside A; 13 = lanatoside D; 14 = lanatoside C; 15 = lanatoside E. Solvent system: *n*-hexane–chloroform–acetonitrile (30:10:9) other conditions as in Fig. 2.

Limits of detection

The 4-NB-derivatives possess high extinction coefficients[13]. The solvents used for the separation do not have any appreciable UV absorption at 260 nm, which results in an excellent signal-to-noise ratio and enhances the sensitivity. Another possibility for improving the detection limits is to increase the volume injected. Up to 100 μl were injected by means of a Rheodyne 7105 injection system with little loss in resolution. The detection limits for some representative glycosides are given in Table IV. The Valco loop injector was used for 25-μl injection volumes and the Rheodyne system for 100 μl. The values are reported at a signal-to-noise ratio of 3:1. It can be seen that only with k' values below 10 a small band broadening is effective. This is the reason why at $k' < 10$ the improvement in detection limit is slightly below the theoretical multiplication factor of 4. A major advantage of the derivatization technique is also that the detection limits do not become inferior for strongly retained derivatives. The last eluted compounds have the highest degree of substitution, *e.g.*,

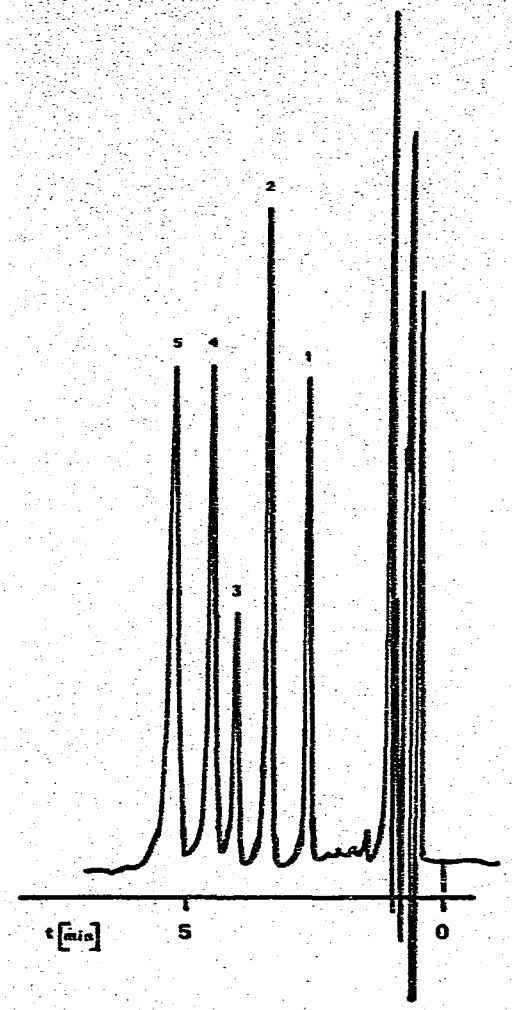


Fig. 4. HPLC of the 4-NB derivatives of five aglycones. 1 = Digitoxigenin; 2 = gitoxigenin; 3 = gitaloxigenin; 4 = digoxigenin; 5 = diginatigenin. Solvent system: *n*-hexane–methylene chloride–acetonitrile–methanol (35:6:2.5:1). Flow-rate: 1.75 ml/min; p = 120 atm. Other conditions as in Fig. 2.

desacetyl lanatoside C is 8-fold substituted in contrast to digitoxigenin, which is only monosubstituted. Calibration functions have been studied previously[13] and the graphs were observed to be linear down to the lower nanogram concentration region and all passed through the origin.

Application to pharmaceutical preparations

The method was applied to the determination of desacetyl lanatoside C and three of its accompanying glycosides in ampoule solutions (Table V). The derivatization of the ampoule extract is described under Experimental section. The injection of the samples was effected via a loop with a 25-μl volume. The solvent mixture *n*-

TABLE IV

DETECTION LIMITS FOR SOME DIGITALIS GLYCOSIDE 4-NB DERIVATIVES TAKEN AT A 3:1 SIGNAL-TO-NOISE RATIO

(1) Volume 25 μl, Valco valve; (2) volume 100 μl, Rheodyne 7105 injection system. (1)/(2), Factor for the increase in sensitivity due to the 4-fold increase in injection volume. Conditions: solvent, *n*-hexane–methylene chloride–acetonitrile (10:3:3); flow-rate, 1.5 ml/min; column, SI 60, 5 μm, 15 cm × 3 mm I.D. Apparatus: Hewlett-Packard UFC 1000; detector, DuPont 842, $\lambda = 254$ nm; maximum amplification.

4-NB derivative	*4-NB groups*	*k'*	*Detection limit (ng/ml)*		*(1)/(2)*
			(1)	*(2)*	
Digitoxigenin	1	3.87	42.4	13.4	3.16
Digoxigenin	2	5.66	26.0	7.3	3.56
Digitoxin	4	9.14	45.6	11.1	4.11
Digoxin	5	11.9	42.8	11.0	3.89
Lanatoside A	6	19.1	64.8	15.8	4.10
Lanatoside C	7	24.6	78.0	19.3	4.04
Desacetyl lanatoside C	8	28.9	78.4	19.6	4.00

TABLE V

ACTIVE SUBSTANCE AND DEGRADATION PRODUCTS IN ONE AMPOULE

100% = 400 μg of desacetyl lanatoside C (declared amount). Conditions: solvent, *n*-hexane–methylene chloride–acetonitrile (10:3:3); flow-rate, 1.5 ml/min; column, SI 60, 5 μm, 15 cm × 3mm I.D.

Compound	*Concentration per ampoule*	
	μg	*% (calculated as desacetyl lanatoside C)**
Digoxigenin	14.56	8.77
Digoxigenin monodigitoxoside	10.61	4.79
Digoxigenin bisdigitoxoside	14.38	5.19
Desacetyl lanatoside C	304.9	76.2

* Each value is the average of two determinations.

hexane–methylene chloride–acetonitrile (10:3:3) was used at a flow-rate of 1.5 ml/min. The active compound desacetyl lanatoside C was analyzed in seven ampoules of the same batch, and 94.8% of the declared amount was found with a relative standard deviation of 2.2%. One separation took 15 min. The same batch was also analyzed by TLC followed by extraction and reaction with picric acid[2]. The result for 15 ampoules was 95% desacetyl lanatoside C. The agreement between the two methods is reasonable but the HPLC technique offers considerable savings in time and sample.

The analysis of accompanying glycosides was carried out on the same batch. Desacetyl lanatosides A and B and lanatoside C were of interest. The chromatography was carried out as described above and 50-μl injection volume was used. The amplification was increased 8-fold. Calibration was effected by external standardization. The following values were found for a set of seven analyses: desacetyl lanatoside B, 2.01 μg, relative standard deviation 5.0% (0.5% of the declared active substance); desacetyl lanatoside A, 0.80 μg, relative standard deviation 10.6% (0.2% of the de-

clared active substance). The concentration of lanatoside C was below 0.1% of the concentration of the active substance desacetyl lanatoside C.

CONCLUSION

The stable 4-nitrobenzoates of the digitalis glycosides are suitable for the trace analysis of these pharmaceutically interesting group of compounds. The separation properties are improved by the derivatization step. The polarities are reduced and the solubilities become more similar without a decrease in selectivity. This effect offers the possibility of carrying out rapid isocratic separations of complex mixtures as they are usually encountered in glycoside extracts and pharmaceutical products. With silica gel as the stationary phase, relatively low-viscosity, low-polarity solvents can be used. The high extinction coefficients of the derivatives and the favourable wavelength of the absorption maximum permits convenient and sensitive detection. Detection limits are below 20 ng/ml provided that an optimal injection volume of 100 μl is used.

As a practical application, it has been shown that the active substance can be analyzed reliably in one ampoule. The major advantage, however, is the sensitivity of the method, which easily permits the determination of by-products and degradation products down to 0.1% of the total glycoside concentration in a single ampoule. Applications to solid pharmaceutical forms and dry extracts were also very promising. The time needed for derivatization and extraction is less than 1 h and the separation takes less than 15 min, which makes this method particularly advantageous for serial analysis.

The 4-NBCl derivatization technique should be applicable to other groups of compounds such as sugars, amino sugars and alcohols, which are not UV absorbing. Such investigations are currently in progress.

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