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A COMPARISON OF REVERSED-PHASE AND PARTITION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SOME DIGITALIS GLYCOSIDES*

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SUMMARY

Comparison of the data for adsorption and reversed-phase chromatography of digitalis glycosides shows the complementary nature of the two modes of separation. The correct choice for a particular problem should make possible a rapid and good separation with simple isocratic systems.

Detection limits vary between 10 and 100 ng per injection and permit the analysis of by-products even in low-dosage pharmaceutical formulations. Quantitation is easily possible with both chromatographic techniques using external standardization. The reproducibility for repetitive chromatograms is about 1% relative standard deviation for manual injections by loop injectors and is even significantly better for automatic injection.

Reversed-phase chromatography can offer some advantages with regard to sample preparation of pharmaceutical formulations.

INTRODUCTION

Digitalis glycosides of the cardenolide group are important pharmaceutically active substances for the treatment of heart disease, and are extracted from the digitalis plants as complex mixtures. Even the purified products can therefore contain trace amounts of by-products, which can be analyzed only by the use of powerful separation techniques such as high-performance liquid chromatography (HPLC).

The problems connected with the analysis of digitalis glycosides and the various methods in use for this purpose have been discussed[1]. Recently, the adoption of HPLC has been considered. Evans[2] has discussed some of the difficulties involved in carrying out separations using adsorption chromatography, including the wide range of polarities of the glycosides and by-products and the relatively poor chromophore

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attributed to the butenolide ring of the steroid portion, with $\lambda_{max.}$ at 220 nm. Lindner and Frei[3] have proposed liquid–liquid systems with a choice of solvent systems that have low absorbance at 220 nm. A modification of both detection techniques and chromatographic properties has been achieved by use of benzoylation reactions on the non-aromatic hydroxyl groups of the steroids and glycosides[4,5]. The usefulness of adapting reversed-phase systems to the analysis of such systems has been reported by Castle[6]. Based on our experience we can say that both adsorption and reversed-phase systems have useful areas of application for determination of cardiac glycosides because of the wide range of polarities of the latter. A critical comparison of these two modes has therefore been attempted in this work.

EXPERIMENTAL

Reagents

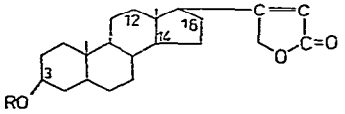
The digitalis glycosides investigated are given in Table I. At least analytical-grade chromatographic solvents and in some instances Uvasol grade (E. Merck, Darmstadt, G.F.R.), were used. The supports used were LiChrosorb Si 60, 10 or 5 μm particle size (Merck), and Nucleosil C_{18} reversed-phase material, 10 μm particle size (Macherey, Nagel & Co., Düren, G.F.R.). Columns were packed according to described slurrying procedures[7,8]. Occasionally, a pre-packed reversed-phase column C_{18} from Waters Assoc. (Milford, Mass., U.S.A.) was used.

Apparatus

Chromatography was carried out with pneumatic pumps, type Haskel (Haskel, Burbank, Calif., U.S.A.). A Perkin-Elmer Model LC 55 detector (Norwalk, Conn., U.S.A.) was used. Also used were Altex 2000 p.s.i. injection valves (Altex Scientific, Berkeley, Calif., U.S.A.) and a Hewlett-Packard automatic sampler HP 7671 A.

TABLE I

CHEMICAL STRUCTURES OF SOME DIGITALIS GLYCOSIDES AND AGLYCONES INVESTIGATED



Compound	*Series*	*Substituent*			
		12	*14*	*16*	*R**
Digitoxigenin	A	H	OH	H	H
Gitoxigenin	B	H	OH	OH	H
Digoxigenin	C	OH	OH	H	H
Digoxin	C	OH	OH	H	D–D–D–
Lanatoside C	C	OH	OH	H	G–AcD–D–D–
Desacetyllanatoside C	C	OH	OH	H	G–D–D–D–
Diginatigenin	D	OH	OH	OH	H
Gitaloxigenin	E	H	OH	–O–C–H (O)	H

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

Integration was effected with a Laboratory Data System 3352 B (Hewlett-Packard, Palo Alto, Calif., U.S.A.).

Sample preparation

The samples and standards were dissolved in methanol or mobile phase and the solutions injected into the chromatograph. Ampoule and drop solutions were injected directly. Tablets were pulverized, the powder being made into a slurry with mobile phase and the clear solution obtained after centrifugation or filtration injected.

RESULTS AND DISCUSSION

Chromatography

The groups with various polarities [aglycones, tridigitoxides and lanatosides (see Table I)] were first studied separately. Fig. 1 shows the separation of the aglycones

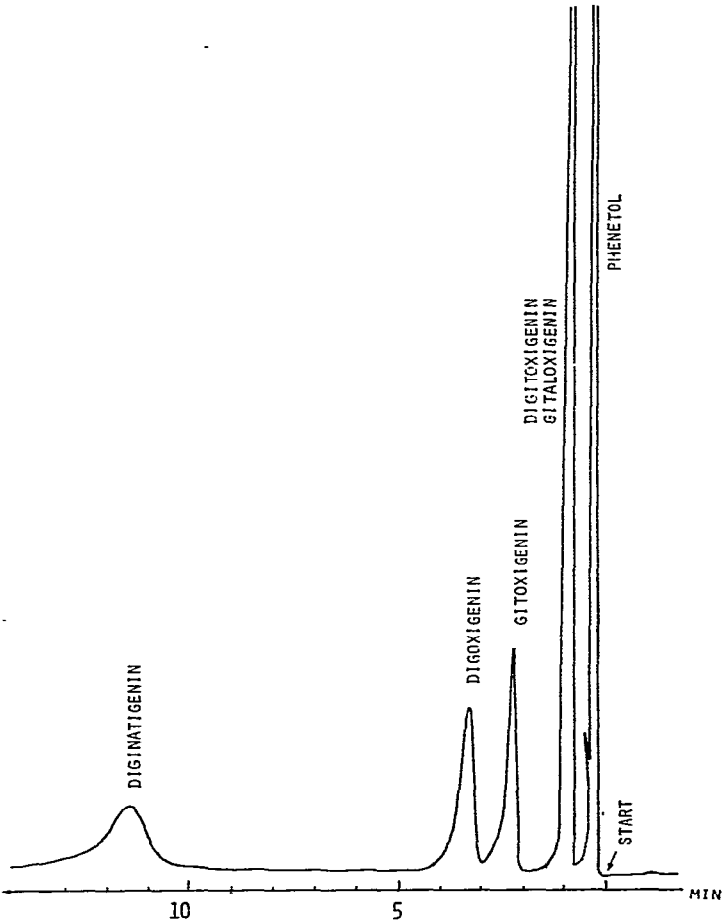


Fig. 1. Separation of some aglycones by adsorption chromatography. Column: LiChrosorb Si 100, particle diameter 10 μm; length 25 cm; I.D. 3 mm. Mobile phase, 3.7% methanol in methylene chloride saturated with water; flow-rate, 2.4 ml/min (Δp 70 bar); injection, 5-μl syringe; detection wavelength 230 nm.

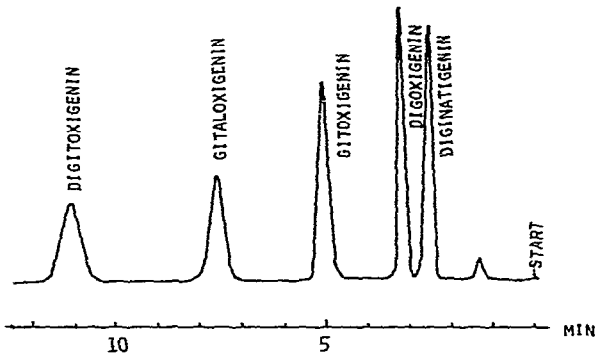


Fig. 2. Separation of some aglycones by reversed-phase chromatography. Column: Reversed phase C_{18}, particle diameter 10 μm; length 30 cm; I.D. 3.5 mm. Mobile phase, 37% acetonitrile (Uvasol) in water; flow-rate, 1.4 ml/min (pressure 100 bar); injection, 25-μl loop; detection wavelength 220 nm.

by adsorption chromatography. As expected, the order of separation follows the order of increasing polarity (Table I), with the pair digitoxigenin–gitaloxigenin not being resolved.

For reversed-phase separations (Fig. 2) the order is exactly reversed, with the digitoxigenin and gitaloxigenin being completely resolved in the expected order. For this separation a simple binary solvent system (a 37% solution of acetonitrile in water)

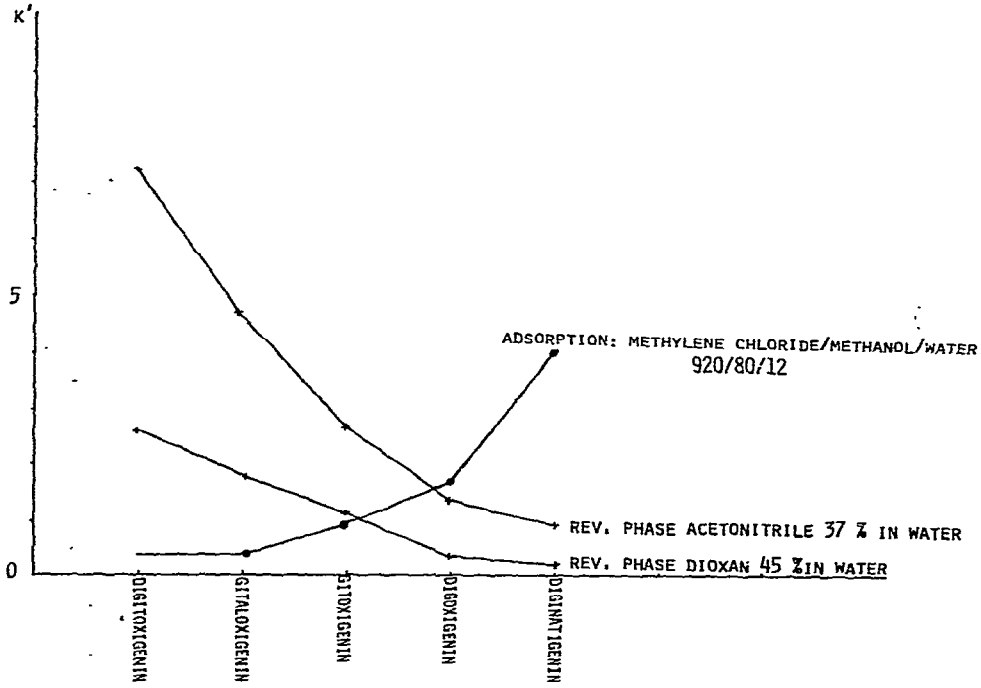


Fig. 3. Retention of aglycones; comparison of adsorption and reversed-phase systems.

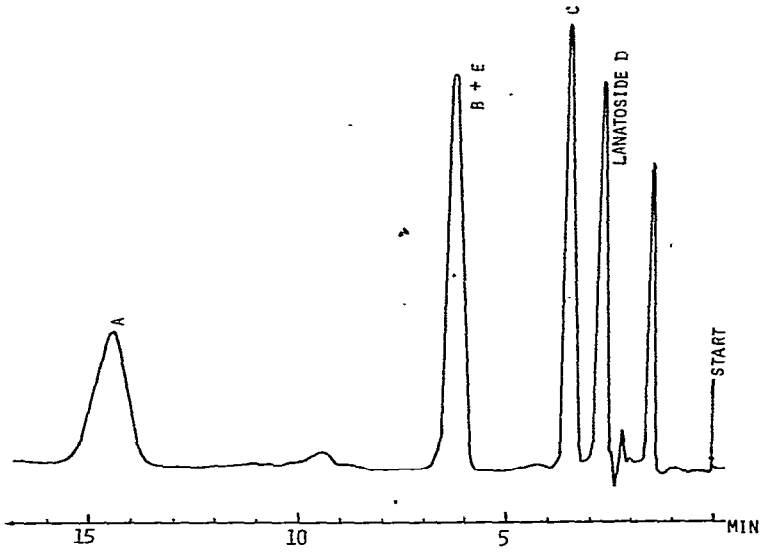


Fig. 4. Separation of lanatosides by reversed-phase chromatography (conditions as in Fig. 2).

was applicable. The retention values of the five aglycones are compared in Fig. 3 for two reversed-phase systems and one adsorption system. The reversibility is clearly demonstrated. A separation of the lanatosides (R = G-AcD-D-D) is shown in Fig. 4. The separation of E and B is difficult to achieve in both adsorption and reversed-phase systems, as can also be seen in Fig. 5. There is some doubt, however, as to the identity of lanatoside E in the chromatogram, as the lanatoside E is known to be easily converted into lanatoside B. Otherwise, the orders of separation in both the adsorptive and reversed-phase modes follow the relative polarities of the molecules and are truly reversed. The behaviour of the desacetyllanatosides is similar.

Fig. 6 shows a complete separation of the tridigitoxosides (R = D-D-D), which should be somewhat intermediate in polarity between the aglycones and the lanatosides of the appropriate families. The separation of the two pairs digitoxin–gitaloxin and digoxin–gitoxin, which is difficult to achieve by adsorption chromatography, is excellent in the reversed-phase mode (Fig. 7).

From the k' values reported in Table II for adsorption conditions, and for several solvent systems with reversed-phase conditions, it can be seen that in comparison with adsorption chromatography reversed-phase chromatography does not give as good a separation of the different groups (aglycones, tridigitoxosides, lanatosides and desacetyllanatosides). Considerable overlapping occurs, which can make the separations of members of one family difficult. An example is given for the separation of the components of the C series. A good separation of the four components by adsorption chromatography is shown in Fig. 8. With the reversed-phase mode, using a mixture of water and acetonitrile on a C_{18} support, the separation of lanatoside C from its aglycone digoxigenin becomes difficult (Fig. 9). In addition, it can be observed that the order of elution is not exactly reversed; digoxigenin elutes first in both modes.

A satisfactory separation of lanatoside C from digoxigenin can be achieved by replacing acetonitrile with dioxan (Fig. 9), but then digoxin starts to interfere. The

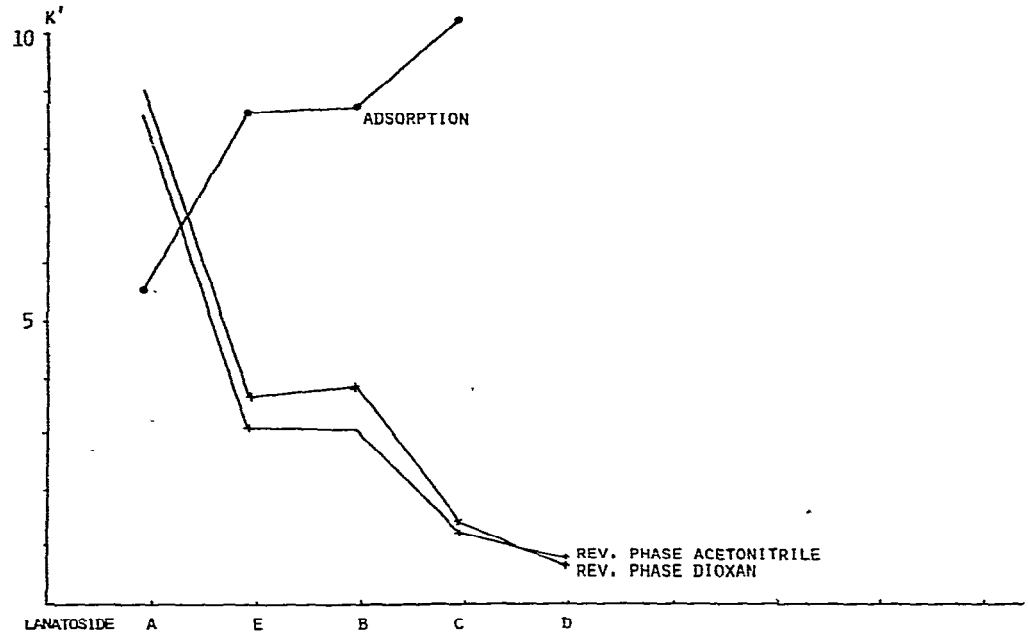


Fig. 5. Retention of lanatosides; comparison of adsorption and reversed-phase systems (reversed-phase solvents as in Fig. 3).

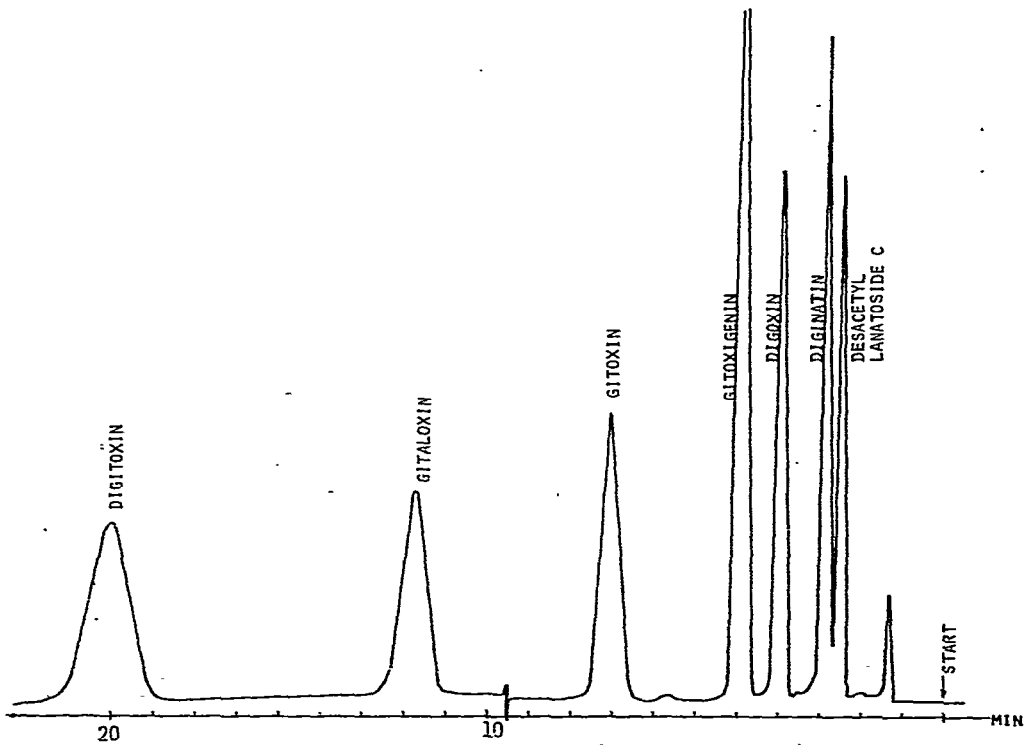


Fig. 6. Separation of tridigitoxosides by reversed-phase chromatography (conditions as in Fig. 2).

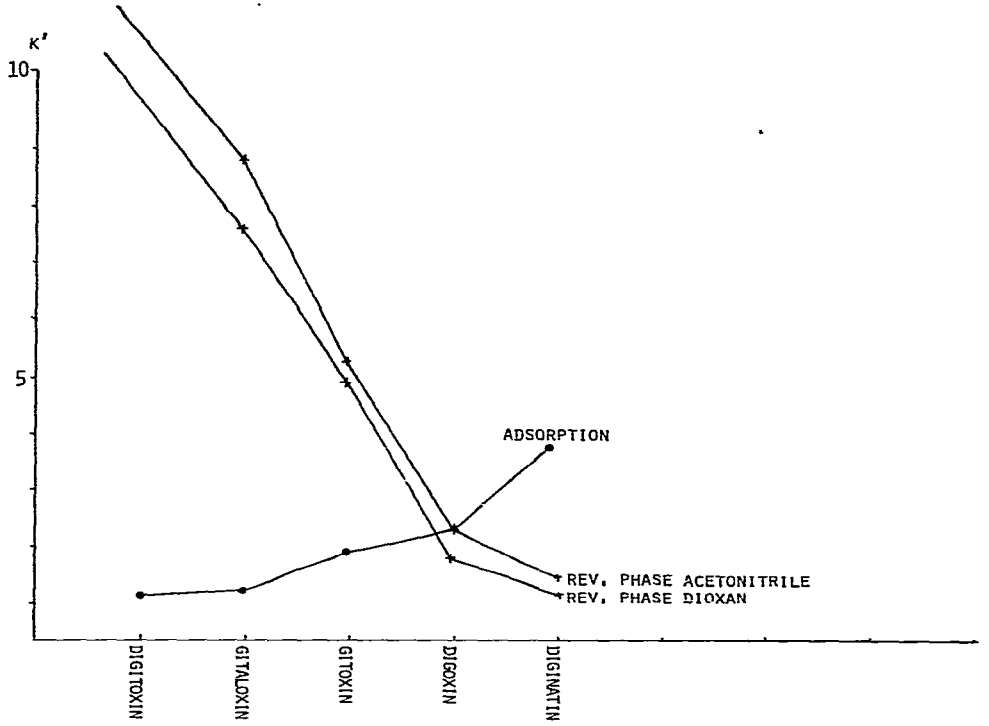


Fig. 7. Retention of tridigitoxosides; comparison of adsorption and reversed-phase systems (reversed-phase solvents as in Fig. 3).

TABLE II

COMPARISON OF k' VALUES FOR DIGITALIS GLYCOSIDES SEPARATED BY AN ADSORPTION AND REVERSED-PHASE SYSTEM

Compound	*Adsorption (see Fig. 12)*	*Reversed phase*		
		37% acetonitrile in water	*45% dioxan in water*	*Tetrahydrofuran–dioxan (2:1 v/v); 33% water*
Digitoxigenin	0.35	7.15	2.55	3.77
Gitoxigenin	0.91	2.65	1.10	2.20
Digoxigenin	1.56	1.30	0.30	1.14
Diginatigenin	3.9	0.85	0.17	0.63
Gitaloxigenin	0.35	4.60	1.70	2.64
Digitoxin	0.76	13.80	11.10	7.77
Gitoxin	1.55	4.90	4.56	4.21
Digoxin	1.90	1.95	1.43	1.65
Diginatin	3.40	1.10	0.80	1.10
Gitaloxin	0.86	8.40	7.20	8.00
Lanatoside A	5.50	8.60	9.00	9.52
Lanatoside B	8.71	3.10	3.85	4.89
Lanatoside C	11.27	1.30	1.18	1.99
Lanatoside D	13.40	0.80	0.70	1.16
Lanatoside E	8.60	3.10	3.70	4.54
Desacetyllanatoside C	15.40	0.90	1.40	1.41

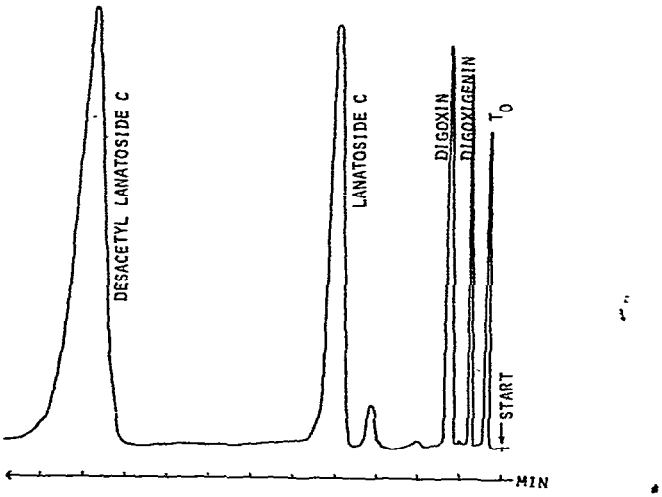


Fig. 8. Separation of the C series of the digitalis glycosides by adsorption chromatography. Column: LiChrosorb Si 60, particle diameter 5 μm; length 15 cm; I.D. 3 mm. Mobile phase, 8% methanol in methylene chloride saturated with water; flow-rate, 2.0 ml/min (Δp 200 bar); injection, 30 μl; detection wavelength 230 nm.

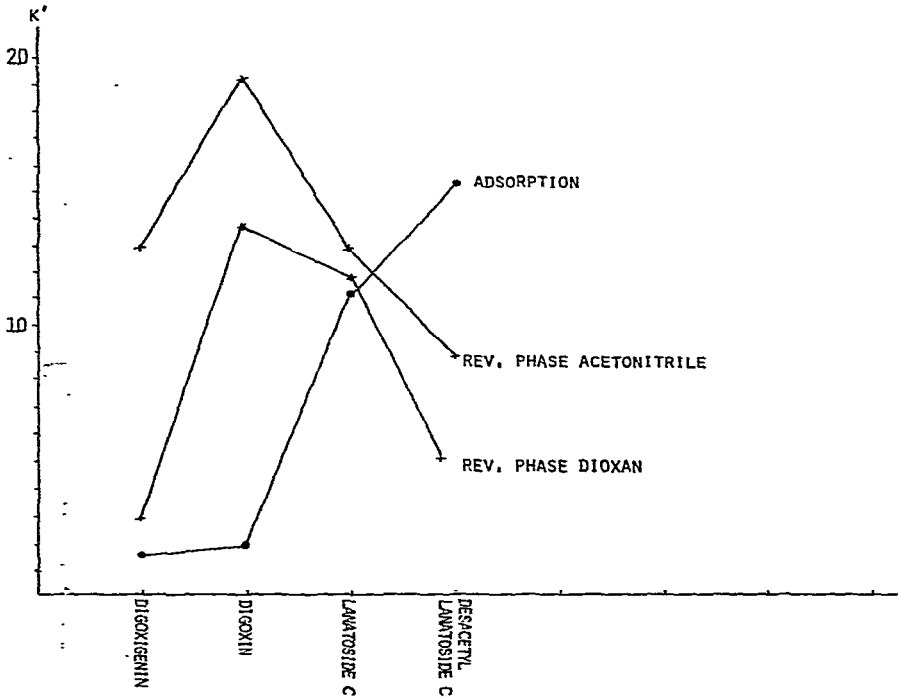


Fig. 9. Retention of the C series of the digitalis glycosides; comparison of reversed-phase and adsorption chromatography (reversed-phase solvents as in Fig. 3).

best compromise was obtained with a mobile phase consisting of a solution of equal amounts of acetonitrile and dioxan in water (Fig. 10). Good results were also obtained by substituting tetrahydrofuran for acetonitrile, as can be seen in Table II.

Reproducibility

The reproducibility for repeated injections has been found to be dependent on the injection device used. For manual injection we recommend the use of loops, which permit a reproducibility of about 1 % relative standard deviation. Automatic injection via septa using a gas chromatographic auto-injector can lead to a reproducibility of $< 0.5\%$ relative standard deviation. Owing to this excellent reproducilility external standardisation techniques have mostly been used. For manual injection via septa or stop-flow techniques, however, the use of an internal standard is recommended.

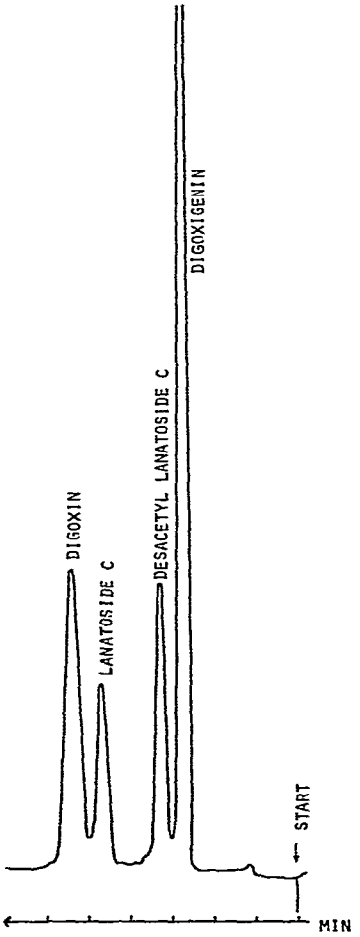


Fig. 10. Separation of the C series of the digitalis glycosides by reversed-phase chromatography. Mobile phase, a 40% solution of acetonitrile–dioxan (1:1) in water; flow-rate, 1.3 ml/min (Δp 140 bar); other conditions as in Fig. 2.

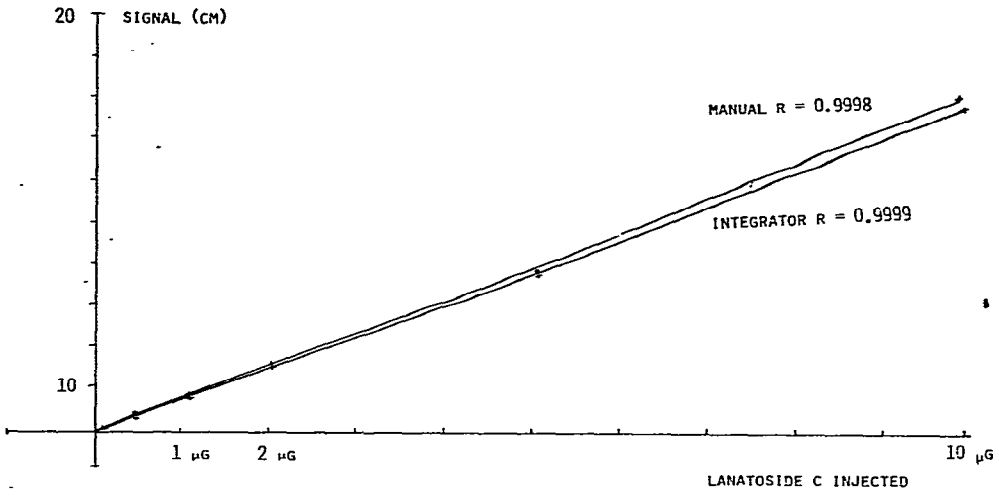


Fig. 11. Calibration curves for lanatoside C.

Calibration curves

It has generally been found that calibration curves are linear over at least a 100-fold concentration range and extend through the origin. The curves for lanatoside C ($k' \approx 11$) are given in Fig. 11 with the coefficients of regression. The entire dynamic range down to the detection limit of 100 ng per injection can be used for quantitative purposes.

Detection limits

As the absorption maximum of the butenolide ring occurs at about 220 nm, the ultraviolet absorption of the chromatographic solvents becomes a determining factor for the sensitivity of detection[3]. Further, because the chromophore is constant throughout, one would expect molar absorptivities to be identical for all the glycosides tested, and, if converted into nanograms per injection, they would consequently be highest for the aglycones and lowest for the lanatosides. Another factor to be considered is the chromatographic dilution process, which becomes significant for substances with high k' values.

As can also be seen in Fig. 11, the detection limit for lanatoside C (signal to noise ratio 3:1) is about 100 ng per injection, and for the digoxigenin with $k' \approx 1$, 37 ng per injection, the injection volume being in both instances 10 μl. In reversed-phase chromatography the transparency of the solvents is greater, which permits detection at 220 nm, and as a consequence, the detection limits can improve from 2- to 4-fold in a reversed-phase system. Additional gains can be made by using higher injection volumes; up to 350-μl loops have been used.

Applications

Adsorption chromatographic techniques have been used with considerable success for the analysis of batches of drug substances, the samples being dissolved in the mobile phase and the solutions obtained injected. Fig. 12 shows an analysis of lanatoside C and by-products. About 1% digoxigenin (corresponding to about 100

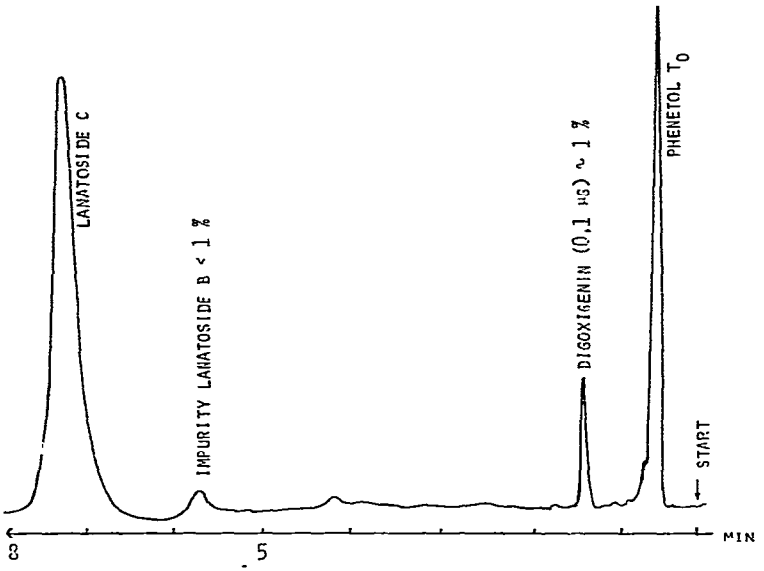


Fig. 12. Analysis of lanatoside C and by-products. Column: LiChrosorb Si 100, particle diameter 5 μm; length 15 cm; I.D. 3 mm. Mobile phase, methylene chloride–methanol–water (920:80:12); flow-rate, 1.2 ml/min; injection, automatic injector HP 7671 A, 10-μl syringe; detection wavelength 230 nm.

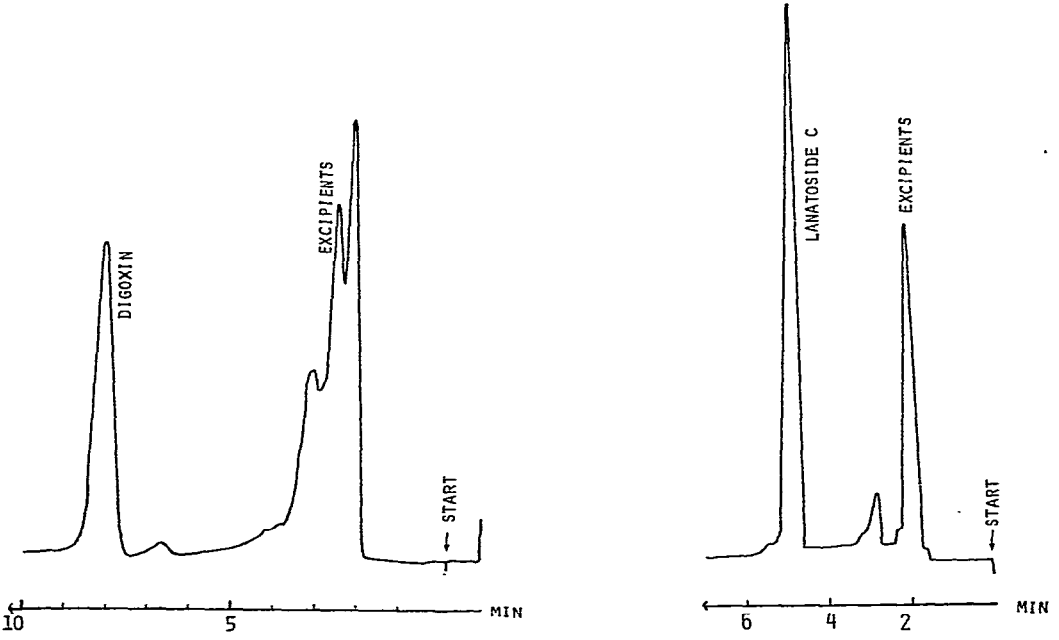


Fig. 13. Analysis of digoxin in a drop solution by direct injection of the solution on to a reversed-phase system (conditions as in Fig. 2).

Fig. 14. Analysis of lanatoside C in a tablet by reversed-phase chromatography. Slurry of crushed tablet with mobile phase, supernatant liquid injected (conditions as in Fig. 2).

μg per injection) was analyzed with a relative standard deviation of 2.8% (N = 12). The problems with the use of adsorption systems for the analysis of dosage forms lie in the sample preparation, and complex extraction procedures have to be used in order to obtain a quantitative recovery of the digitalis glycosides. On the other hand, the greater solubility of these compounds in the relatively polar solvents used in reversed-phase chromatography enables sample preparation to be simplified.

Injection and drop solutions can be applied directly on to the column, as can be seen in Fig. 13. Excipients are partially eluted with the solvent front or with a k' value that is sufficiently small to permit separation from the peak of the drug substance itself. Tablet formulations can be analyzed by making a slurry of the crushed tablet with the mobile phase, centrifuging it and injecting the clear solution on to the column. The results of such an analysis can be seen in Fig. 14.

CONCLUSIONS

For the separation of digitalis glycosides, adsorption and reversed-phase chromatography fulfil somewhat complementary functions. As true reversibility of orders of separation is observed, one can, in most instances, choose the mode that is best suited to the particular problem and requirements, *i.e.*, saving of separation time and increasing resolution. By choosing the appropriate mode, separations can be carried out isocratically and at room temperature with simple chromatographic systems that are easy to operate. Reversed-phase systems seem to have advantages in regard to sample preparation for analysis of dosage forms. The slightly improved sensitivity in these latter systems can be of importance when chromatographing low-dosage pharmaceutical forms or for the quantitation of by-products.

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