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Fluorescence detection of cardenolides in reversed-phase high-performance liquid chromatography after post-column derivatization

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Abstract

Methods for the fluorescence derivatization of cardiac glycosides with concentrated acids from TLC are adopted to HPLC for post-column derivatization. The column effluent is blended with concentrated acids in a knitted tube reactor, which enables derivatization with negligible increase in chromatographic peak width. The selectivity of the reaction is temperature-dependent and influenced by the respective acid. Reactivity increases from $\text{H}_3\text{PO}_4 \rightarrow \text{CH}_3\text{SO}_3\text{H} \equiv \text{H}_2\text{SO}_4$. The conversion of digoxigenin, digitoxigenin and their digitoxosides is accelerated by Cu(II) acetate or Co(II) nitrate in H_2SO_4 . Combined with a new two-mode, single-column solid-phase sample preparation, cardiac glycoside levels of less than 100 pg/glycoside in 1 ml plasma are detectable.

Keywords: Cardenolides; Glycosides; Digoxin

1. Introduction

Cardiac glycosides are the classical drugs [1] for the treatment of congestive heart failure. High-performance liquid chromatography has been proven as a selective and sensitive instrument for the determination of these drugs, especially since miniaturization of the system by Fujii et al. [2], when a lower detection limit of 1 ng/0.1 μl was achieved. However in physiological media, cardenolides like digoxin are actually not amenable to chromatographic detection methods at therapeutic concentrations in the low ng/ml range [3]. Clinically they are quantified exclusively by immunological methods, coupled to different detection systems (RIA, ELISA, FPIA). Even recent publications [3,4], introducing an

additional chromatographic step to avoid the cross-reactivity to other physiological steroids, depend on the immune response detection principle, coupled on- or off-line to HPLC systems. These combinations are valuable for the investigation of drug metabolism, physiological research, and for the identification of substances interfering with immunological assays, but seem too complex for clinical routine use. They were successfully applied for the isolation of endogenous and exogenous digitalis-like factors (DLFs) from various sources [5–8], but it can be argued that the inherent loss in chromatographic resolution caused by these combinations is too large for the separation of closely related molecules, such as the ouabain-like factors (OLF) [9–11] or digoxin-like factors (DxLF), as the OLF is discussed to be extremely similar to, but not identical with ouabain [10,12]. If the speculations about the steroidal nature

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of these factors are proven, an analytical method for digitaloids should distinguish endogenous and exogenous factors irrespective of their pathophysiological significance [13] without the handicap of non-linear response of the gene–antigen reactions.

For quality control purposes, steroids and cardenolides have been measured with excellent results and simplicity in plant extracts and drug formulations with TLC [14]. Sensitive detection was achieved by post-chromatographic exposition of the TLC plates to acids and subsequent fluorescence monitoring. HPLC separations with UV detection have been published [15], but with insufficient sensitivity. However, successful transfer of TLC derivatization reactions to continuous flow reactors in HPLC systems as for amino acid analyses has also been proved for cardenolide detection [16–19] using concentrated hydrochloric acid for derivatization. Comparable results were obtained in TLC derivatization with orthophosphoric acid and sulfuric acid, which do not develop corrosive gases and are therefore potentially more convenient as reaction additives. This point gains importance, if derivatization at enhanced temperatures is needed, allowing a reduction of the required residence time in the reactor. In parallel, viscosity and backpressure may be reduced and thus sensitivity should increase.

Therefore the first aim of our work was a detection method, which can be coupled on-line to HPLC systems with sufficient sensitivity for determinations in the range down to 0.5 ng per injection and, if possible, for pharmacokinetic purposes as low as 0.1 ng per injection or less. The diameter of the column was chosen as low as possible for routine applications (2 mm I.D.), coupled to a reactor with minimal band broadening (knitted PTFE tube, 0.3 mm I.D.).

The optimized analytical system was then used to develop a selective solid-phase extraction scheme for digoxin and some of the corresponding metabolites from physiological fluids. Liquid–liquid extraction of digoxin [20] seems rather laborious and inconvenient for large series. However, sample clean-up of digoxin (nmol/l) from the complex matrix of endogenous steroids affords an extremely selective SPE sequence and could not be accomplished by a simple adsorption–wash–desorption cycle. A scheme was developed, utilizing both lipophilic (reversed-phase mode) and polar interactions (normal-phase mode) of

the solutes with a single column packing. In two experiments digoxin levels were measured in plasma or serum samples. The first series was collected from male healthy volunteers after a single oral dose, and the second included a group of elderly, hospitalized patients under regular digoxin regimen. Results from the latter are compared with digoxin levels obtained from routine fluorescence polarization immunoassays.

2. Experimental

2.1. Chemicals

Methanol (MeOH) and acetonitrile (MeCN) in chromatographic gradient grade quality, and ethylacetate (EtAc), hexane and acetone in chromatographic grade were purchased from Merck (Darmstadt, Germany). Sulfuric acid (~96%), orthophosphoric acid (~85%), trifluoroacetic acid (TFA), hydrochloric acid (~35%), nitric acid (~60%) (analytical grade) and methanesulfonic acid (practical grade) were from Merck. Metal salts were purchased in analytical grade quality from Merck. For calibration purposes plasma from healthy donors was purchased from the Deutsches Rotes Kreuz Blutspendedienst (Ulm, Germany).

2.2. Equipment

A ceramic spotting plate was placed on a normal heating plate to screen the reaction conditions. Fluorescence was induced with a hand held UV lamp with two switchable wavelengths (excitation at 360 nm).

Off-line fluorescence spectra were recorded with a spectrofluorimeter equipped with two double monochromators (PTI Photon Technology, Wedel, Germany). The HPLC post-column setup is shown in Fig. 1. Eluents were delivered by a low pressure gradient LC 9A pump (Shimadzu, Duisburg, Germany). Samples were injected via a Rheodyne 7125 injection valve equipped with a laboratory filled pre-column 25×4 mm (LiChrolut RP18, 40–63 μm, Merck) for the injection loop. All chromatographic separations were performed on a 100×2 mm HPLC

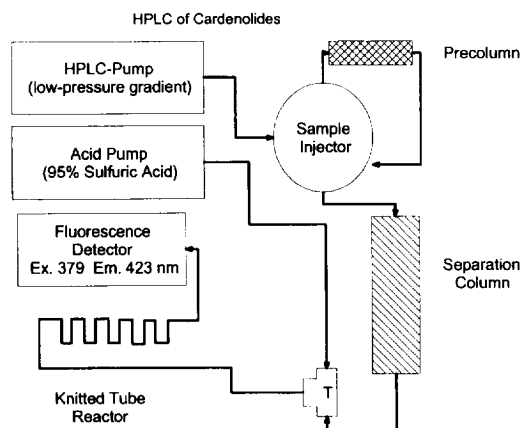


Fig. 1. Schematic representation of the HPLC system used for cardenolide analysis with post-column derivatization with 95% sulfuric acid. Dilute samples are concentrated on the precolumn. Backflushing of the precolumn after the start of the solvent gradient focuses the sample for maximal sensitivity.

column (Superspher RP18, 4 μm , Merck). Chromatograms with UV detection and UV spectra were recorded by an M10A diode-array spectrophotometer (Shimadzu). For post-column derivatization, a knitted PTFE-tube (5 m length, 1.6/0.3 mm O.D./I.D.; Marianne Büchele, Kirchberg/Iller, Germany) was coupled to the column outlet by a T-piece. Derivatization acid was delivered by an HPLC pump S1020 with a pump head manufactured from Tefzel (Sykam, Garching, Germany) and fed into the second inlet of the T-piece. The outlet of the tube reactors was connected to an RF551 fluorescence detector (Shimadzu). To avoid corrosion problems, the original flow-through cell retainer was replaced by laboratory made PTFE parts. Temperature stabilization in the reaction capillary was achieved by immersion in a beaker filled with PEG400, placed on a heater with an adjustable contact thermometer and magnetic stirrer. Solid-phase extractions were performed with a VacElut manifold (Merck). SPE-columns were manually packed (200 mg) with LCA-Si06 (Sykam) into 4-ml columns (Baker, Grossgerau, Germany). According to technical information, LCA-Si06 is a short-chain alkyl-silica material. Fluorescence polarization immunoassays (FPIA) were performed with the TDxII assay (Abbott Laboratories, Diagnostics Division, Chicago, IL, USA),

lower limit of determination 0.5 ng digoxin per ml. Superspher and Aluspher are trademarks of Merck.

2.3. HPLC separation conditions for digoxin and metabolites

The sorbent was Superspher RP18 (4 μm , 100 \times 2 mm I.D.). Eluent A was 100% H_2O and eluent B 100% MeCN; the flow rate was 0.2 ml/min. The following gradient was used: 0–0.1 min, 10% B; 0.1–4.0 min, 10–18% B; 4.0–19.0 min, 18–26% B; 19.0–26.0 min, 26–75% B; 26.0–29.0 min, 75–10% B. The flow rate of derivatization medium was 0.35 ml/min. Reequilibration was completed after 44 min.

2.4. Screening of fluorogenic steroids and post-column derivatization

2.4.1. Screening of fluorogenic steroids

A 1-mg amount of each substance was dissolved in 2 ml MeCN. Some were dissolved as far as possible. A 200- μl volume of each solution was pipetted into an empty well and 200 μl of the appropriate acid were added for fluorescence derivatization. Temperature was maintained with a ceramic heating plate adjusted to 80°C. Fluorescence intensity was judged visually after three minutes at room and at elevated temperature.

2.4.2. Post-column reactor

The dependency of peak dispersion and tube length and volume was investigated with three reactors differing in length and I.D. in a model system at different flow rates [column: Aluspher RP, 5- μm , 125 \times 4 mm I.D.; UV detector: cell volume 8 μl ; eluent: 75% (v/v) MeCN- H_2O]. Plate numbers were calculated from the first and second moments of the peak of nitrate and toluene respectively.

$$\mu'_i = t_R = \frac{\sum t_i \times C_i \times \Delta t}{\sum C_i \times \Delta t} \quad (1)$$

$$\mu'_2 = \sigma^2 = \frac{\sum (t_i - \mu'_1)^2 \times C_i \times \Delta t}{\sum C_i \times \Delta t} \quad (2)$$

$$N = (t_R / \sigma)^2 \quad (3)$$

N_{eff} was derived with t'_R instead of t_R .

2.5. Digoxin and digoxin metabolites in human blood samples

Pharmacokinetic investigations of male healthy volunteers ($n=5$) were part of a thesis [21]. Blood samples were taken after an oral single dose of 0.5 mg digoxin. Blood was collected in EDTA tubes. The plasma was separated after centrifugation at 660 g for 10 min and stored frozen at -20°C for analysis. Serum samples from hospitalized patients (age >60 years, $n=24$) were collected as part of another thesis [22]. Results have been presented in parts together with results of digitoxin determinations (data not shown here, modified method) [23]. Results of the HPLC method were compared with TdxII FPIA. For this purpose digoxin and the identified digoxin metabolites were summed after multiplication with their cross-reactivities [24]. Blood conditioned with heparin or citrate was equally suitable for SPE. These are cited with kind permission of the authors.

2.6. Solid-phase extraction of cardiac glycosides: single column, two modes

Fig. 2 gives the scheme for the separation of cardenolides from plasma or serum. Each column is conditioned with 5 ml MeCN and 4 ml H_2O . A 1-ml sample is diluted with 100 μl H_2O and put on the column. After washes with 2 ml MeOH– H_2O , (20:80, v/v), 3 ml H_2O and 2 ml acetone– H_2O (15:85, v/v) the column is sucked dry (end of the reversed-phase mode). Remaining water is extruded with 1.5 ml hexane–EtAc (90:10, v/v) and the column is switched to normal-phase behaviour. After drying with N_2 , solutes are eluted with 1.5 ml hexane–acetone, (60:40, v/v). After drying, samples were dissolved in 300 μl of MeOH– H_2O (20:80, v/v) and completely injected.

2.7. Statistical evaluation of the HPLC-assay

Detection and determination limits were determined according to the German industrial norm (DIN 32645). Standard and plasma samples were

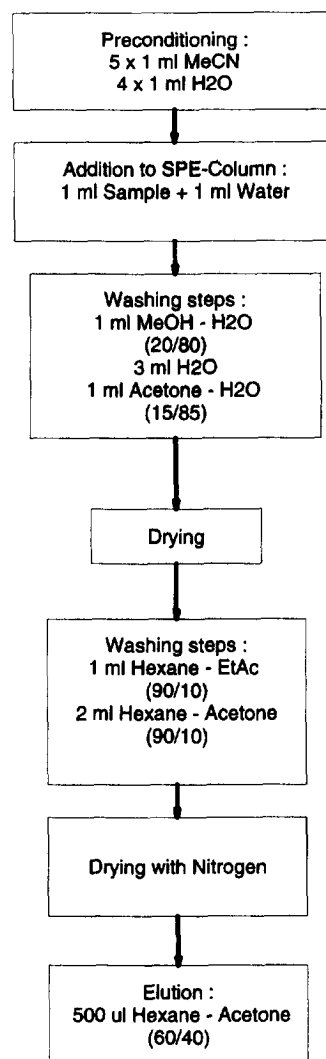


Fig. 2. Solid-phase extraction of cardiac glycosides from plasma or serum samples.

spiked with 12 different concentrations of cardenolides (Table 3) and analyzed in triplicate. Standards [300 μl of 20:80 (v/v) MeOH– H_2O] were injected without extraction. Samples from twenty hospitalized patients without digoxin therapy were analyzed to identify interfering substances. For the same purpose t_R of all steroids from Table 1, of spironolactone, and furosemide were examined.

Table 1
Screening of steroids detectable by fluorescence after addition of concentrated acids

| Substance | H ₃ PO ₄ (80°C) | MSA (RT) | MSA (80°C) |
|---|--|-------------|---------------|
| Oleandrin | + /bl | + /bl | + /bl |
| Proscillaridin | + | + /y | + /y |
| Cymarín | + | + /y-gr | + /y-gr |
| Convallatoxin | + | + /y | + /y |
| Ouabain | + | – | + /y |
| Digitoxin | + /bl | – | + /y |
| β -Sitosterol | – | + /y | + /y |
| Cholic acid | + /bl | + /bl | + /bl |
| Cholesterol | – | + /y-gr | + /y-gr |
| Ethinylestradiol | + /y | + /y | + /y |
| Estrone | + /bl-y | + /bl | + /bl |
| Dehydrocholic acid | + /bl | – | + /bl-gr |
| Deoxycholic acid | + /bl | – | + /bl-gr |
| Chenodeoxycholic acid | + /bl | – | + /bl-gr |
| Glycocholic acid | + | + /bl | + /bl |
| Taurodeoxycholic acid | + | + /bl | + /bl |
| Taurochenodeoxycholic acid | + | + /bl | + /bl |
| Dexamethason | + /y | + /bl-gr | + /bl-gr |
| Dehydroepiandrosterone | + /bl | + /bl-gr | + /bl-gr |
| 4-Androsten-3,17-dione | – | + /bl-gr | + /bl-gr |
| cis-Androsterone | – | + /bl-gr | + /bl-gr |
| Progesterone | – | + /bl | + /bl |
| 5- α -Androsten-17- β -ol-3-OH | – | + /bl-gr | + /bl-gr |
| Aldosterone | – | + /bl-gr | + /bl-gr |
| Nortestosterone | – | + /bl | + /bl |
| Pregnanediol | – | + /bl | + /bl |
| Hydrocortisone | – | + /y | + /y |
| 11-Dehydrocorticosterone | – | + /y | + /y |
| Corticosterone | – | + /bl-y | + /bl-y |
| Pregnenolone | + /bl ^a | + /y | + /y |
| 17- α -Hydroxyprogesterone | + /bl ^a | + /bl | + /bl |

H₃PO₄ = orthophosphoric acid (85%); MSA = methanesulfonic acid; H₂SO₄ = sulfuric acid; y = yellow; bl = blue; gr = green.

^a Compounds reacting with phosphoric acid at room temperature.

3. Results and discussion

3.1. Chromatographic separation of cardenolides

Separation of cardenolides with HPLC has been reported with water–MeCN, water–isopropyl alcohol mixtures on reversed-phase columns for drug formulation testing [15] or for coupling of HPLC to immunoassays [25]. We started with MeCN–water gradients, which have already been reported to give

good separations of cardenolide mixtures [3] on Nucleosil C₁₈, beginning at 20% (v/v) MeCN–water.

In the separation development phase, chromatograms were recorded with an UV diode-array photometer at 220 nm, and the resulting optimal separation conditions (see Section 2) are comparable to the formerly published data on other C₁₈ reversed-phase columns.

3.2. Screening for fluorogenic steroid and post-column derivatization

3.2.1. Screening for fluorogenic steroids

In TLC the generation of fluorescent products from steroids by post chromatographic treatment with strong acids is widely used. Separated steroid spots (cardenolides) are sprayed with concentrated acids (sulfuric acid, p. 411 in Ref. [14]; orthophosphoric, p. 430 in Ref. [14]; hydrochloric acid, p. in Ref. 304 [14]; trichloroacetic acid, p. 420 in Ref. [14]) alone or in combination with vanillic acid or anisaldehyde (p. 195 in Ref. [14]) and fluorescence can be induced (excitation: 360 nm; emission: ~430 nm). While reaction products of the derivatizations are unknown, resuming the reaction conditions, a mixture of conjugated ring systems may be the result. In a screening experiment the efficacy of this reaction for steroid detection in solution was tested. On a ceramic spotting plate several steroids were treated with concentrated acids and fluorescence activity was monitored visually (Table 1). Although the results are only qualitative, obviously the reactivity is strongly dependent on the acid and reaction temperature. Reactivity was highest for methanesulfonic acid at elevated temperatures. In a second experiment sulfuric acid appeared equipotent to MSA. Therefore, and with respect to the easy availability of the pure acid, sulfuric acid was selected for the following experiments.

Fluorescence spectra of cardenolides after batch reaction in acidic solution were recorded, to get approximate wavelength settings for chromatography. Fig. 3 and Fig. 4 display the emission and excitation spectra of digoxin, digitoxin, digoxigenin and digitoxigenin derivative mixtures after acid treatment. The output was corrected by the molar

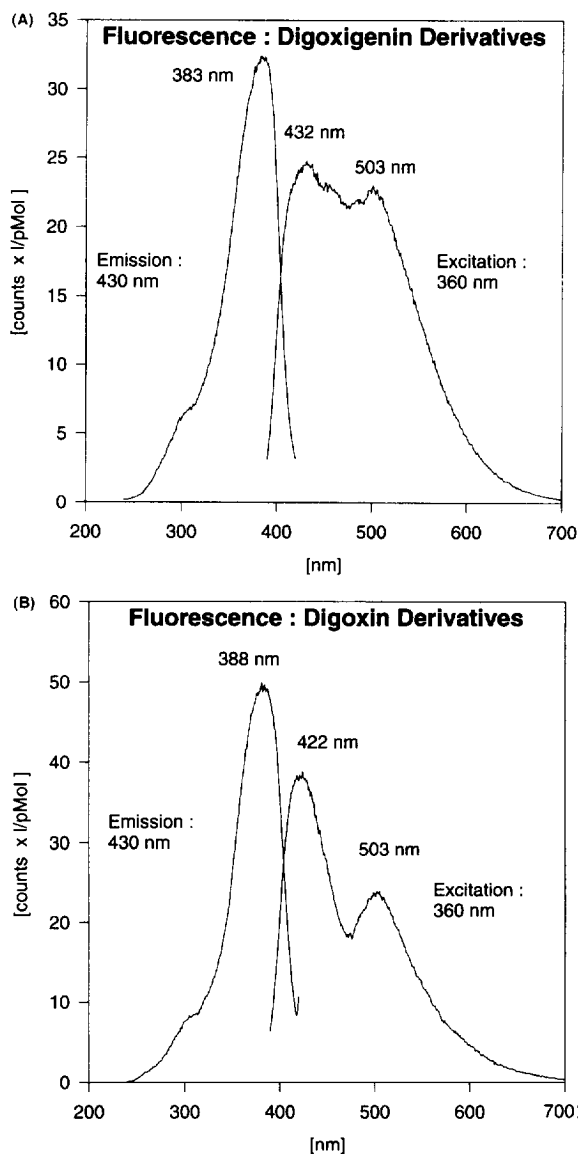


Fig. 3. Fluorescence spectra of digoxigenin derivatives (top) and digoxin (bottom) recorded after batch reaction at room temperature (for reaction conditions, see Fig. 4).

masses to obtain equimolar response. In excitation and emission differences between the genines and the digitoxosides occurred, but should not be over-interpreted, because under these conditions the reaction proceeds slowly and the composition of the cocktail changes. Other results for the relative molar response were obtained under continuous-flow HPLC

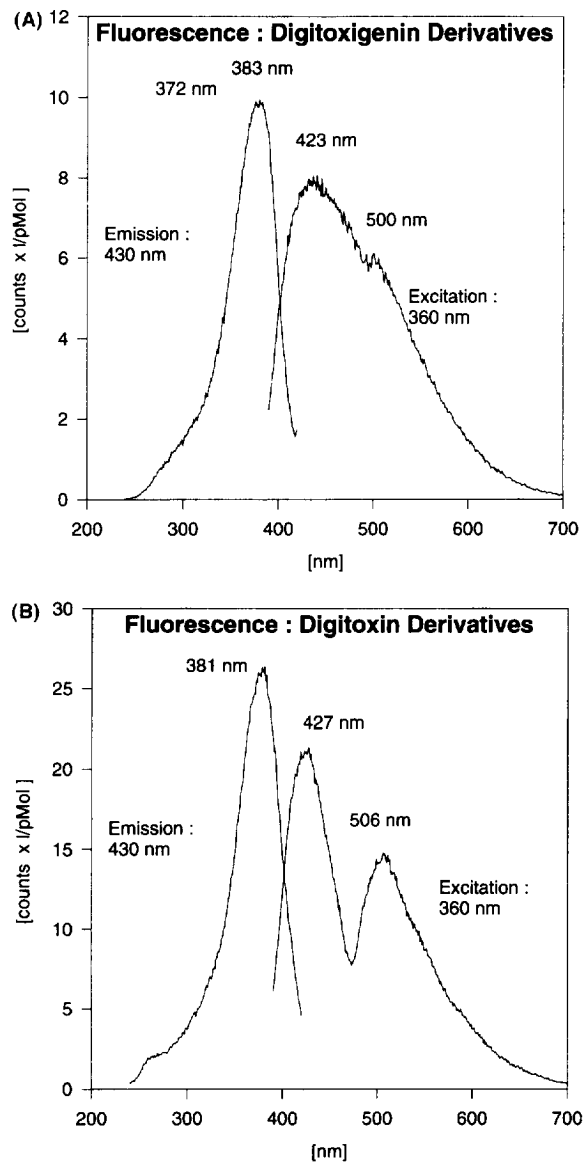


Fig. 4. Fluorescence emission spectra of digitoxigenin derivatives (top) and digitoxin (bottom) recorded after batch reaction at room temperature (M_{exc} 360 nm; 1 nm band width; cell geometry $1 \times 1 \times 1$ cm). A 1-mg amount of substance was dissolved in 10 ml of 60:40 (v/v) MeCN- H_2O . An aliquot of 600 μ l of this solution was mixed in an open vial with 1200 μ l of 95% sulfuric acid. After ~ 1 min reaction time, the emission and the excitation spectra were recorded. Counts correspond to the used cell volume and concentration.

conditions (Fig. 5). These reaction times and conditions are highly reproducible, but compromises between the response of different cardenolides are necessary to obtain optimal conditions for analyses.

Under continuous-flow reactions and with the HPLC fluorescence detector the wavelength settings for the post-column derivatization were controlled. Optimal response for digoxin was found at $\lambda = 379\text{--}423\text{ nm}$ (excitation–emission), which is close to the spectral maximum from the batch experiment. The major reason for the difference is probably the poorer spectral resolution of the HPLC detector of 15 nm.

After installation of the reaction–detection system instead of the diode-array photometer, the reaction temperature for digoxin and the ratio of eluent to acid were optimized stepwise by integration of the peak area. Maximal areas for digoxigenin and its glycosides were obtained at 115°C bath temperature in the 5 m reactor and an optimal eluent/acid flow ratio of 0.2:0.35. The flow ratio proved optimal for all following experiments. Above 115°C the peak area declined. The limit of detection at this temperature was well below 0.5 ng per injection for digoxin (Table 3), but the high temperature above the boiling-point of MeCN was critical. The baseline was disturbed by the high temperature gradient between reactor and detection cell (refractive index fluctuations) and the pressure in the tube reactor increased to ~5 MPa. An additional short restriction capillary held at room temperature after the detector cell was necessary to prevent bubble formation. At lower reaction temperature the disturbances disappeared completely, but the digoxin response diminished simultaneously. A compensation of the lower reaction speed by doubling the length–volume of the capillary tube reactor increased backpressure and peak dispersion considerably without remarkable gain of peak areas, and was therefore only tried once. This result was predictable when the high viscosity of the reaction mixture, which should be fortified at lower temperature, and the normally exponential correlation of reaction speed and temperature are considered.

We tried to lower the reaction temperature by addition of heavy metal ions, known as catalysts for TLC derivatization [14]. At room temperature, digoxin reacts slowly with pure sulfuric acid and even

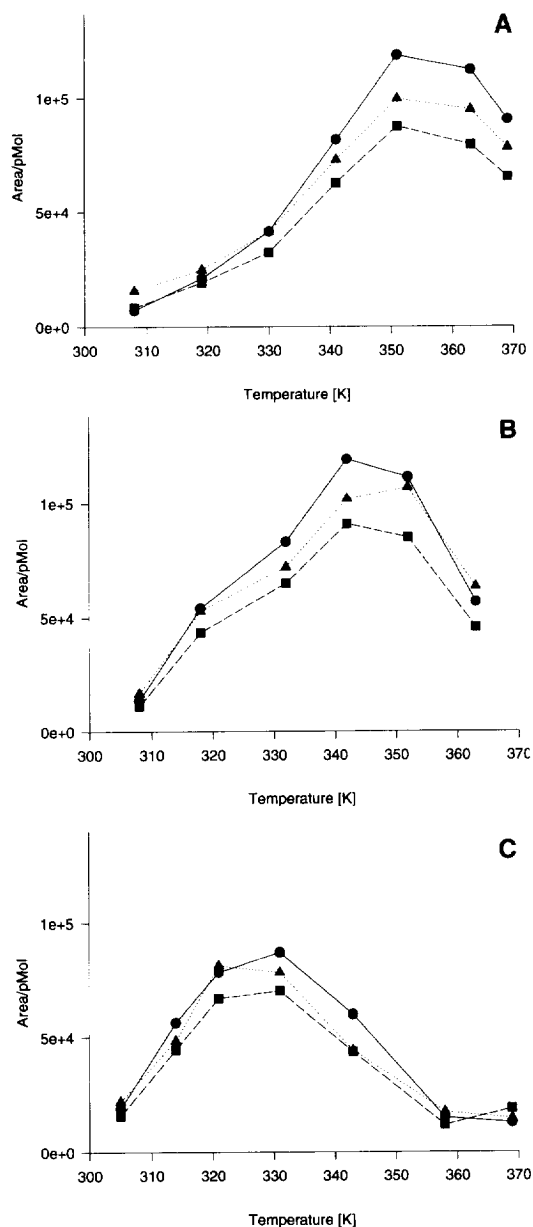


Fig. 5. Optimisation of bath temperature during post-column derivatization at different concentrations of Cu^{2+} acetate in the derivatization acid (95% sulfuric acid) (w/v): A=0.03%; B=0.08%; C=0.4% (total concentration in the reaction mixture: A=0.96 mmol/l; B=2.55 mmol/l; C=12.75 mmol/l); Fluorescence: excitation 383 nm, emission 427 nm). Peak areas/pmol of (▲) digoxin, (■) digoxigeninbisdigitoxoside and (●) digoxigenin were calculated from two injections of 0.9 ng of each cardenolide, respectively. The separation column was removed in this experiment and samples directly injected into the mixing T-piece→knitted tube reactor.

Table 2

Influence of metal ions on the generation of fluorophores from digoxin in the presence of concentrated sulfuric acid and phosphoric acid

| Acid | Ion | | | | | | | | |
|--------------------------------|----------------------------|--|--|--|--|--|--|--|--|
| | Cu ¹⁺ (CuCl) | Cu ²⁺ (CuAc ₂) | Ni ²⁺ (NiSO ₄) | Zn ²⁺ (ZnAc ₂) | Fe ²⁺ (FeCl ₂) | Fe ³⁺ (FeCl ₃) | Co ²⁺ (CoCl ₂) | Mg ²⁺ (MgCl ₂) | Sn ²⁺ (SnCl ₂) |
| H ₂ SO ₄ | 0 | ++ | 0 | 0 | -- | - | ++ | 0 | 0 |
| H ₃ PO ₄ | ++ | 0 | 0 | 0 | -- | - | 0 | 0 | 0 |

0=no effect; - = quenching; -- = strong quenching; ++ = strong increase; concentration of the metal salts: 1% (w/v) acid, other reaction conditions see Section 3.2.

slower with phosphoric acid (conditions in Table 2) and is thus well suited to visualize catalytic effects.

Table 2 presents the influence of some heavy metal salts on fluorophore generation from digoxin in concentrated acids. A remarkable increase was induced by addition of Cu(II)acetate and Co(II)-chloride to sulfuric acid, while with phosphoric acid only Cu¹⁺ promoted fluorescence. Probably the isoelectronic character of the two promoters Cu²⁺ and Co²⁺ is responsible for their similar behaviour in H₂SO₄. The action of Cu(I)chloride in orthophosphoric acid is remarkable given there is a complete absence of activity of Cu²⁺ in this environment. Evidently the acids are not only simple proton donors and water sinks here, but are involved in metal chelate and/or redox reactions.

Unfortunately for an interpretation, additional reactions almost linked to the fluorophore generation occur, and one key parameter, the exact reaction temperature, was not monitored. However, it can be assumed that the eluent-acid mixture immediately heats up by solvation effects of sulfuric acid, water and MeCN, the latter being completely saponified to acetic acid under these conditions. Bath temperatures are therefore far below the maximal reaction temperature. This was underlined by an experiment, to answer the question whether fluorophores may be generated at room temperature in a premixed and cooled MeCN-water-H₂SO₄ cocktail. For this purpose 20 ml of 95% H₂SO₄ were mixed in an open Erlenmeyer flask with 20 ml of 40% (v/v) MeCN-H₂O at room temperature. The whole solution burst out after ~30 s under excessive foaming and development of acetic acid. After cooling, a highly viscous mass was formed, which was easily soluble in water, but was not further analysed. The odour of

MeCN had completely disappeared, which was attributed to the saponification reaction.

Subsequently a reoptimisation of bath temperature at different Cu(II)acetate concentrations was performed, with the reactor of the HPLC-system directly coupled to the injector via the mixing-T. Results are shown in Fig. 5. Optimal response, as a compromise between digoxin response, which is physiologically dominant, and its metabolites, was obtained at 352 K bath temperature at 0.08% (w/v) Cu(II)acetate. Calculated molar responses show small differences (digoxigenin bisdigitoxoside 0.76; digoxin 0.96; digoxigenin 1).

As anticipated, the steroidal part of the molecules plays the dominant role for fluorophore generation, although the relative individual reactivities change with temperature and metal salt concentration. The influence of the substitution at carbon 3 is expressed by the individual response optima and their temperature differences. Obviously the 3 α -hydroxyl is involved in the chromophore generation and the lower response of the *bis*-glycoside may reflect the activation energy to hydrolyse the ether bond, which is partly compensated by an increased contribution of entropy with the larger *tris*-glycoside.

3.2.2. Post-column reactor

Detection of fluorophores generated from steroids in air segmentation reactors has been described [16,17], but in view of the extremely low therapeutic concentrations of cardenolides (digoxin ~1–3 ng/ml human serum) and with respect to the large endogenous pool of steroids, the contribution of this reactor type to peak dispersion does not seem tolerable. Most conventional materials used in HPLC for flow lines, fittings and pump heads rapidly corroded after

contact with the concentrated acid and therefore were manufactured from acid resistant material. PTFE and KelF were used for capillaries and fittings and Tefzel for the pump head respectively (i.e. the original stainless steel retainer of the fluorescence detector cell was leaky after ~one week and severely damaged). Knitted PTFE capillary was used as post-column reactor, combining inertness and low band broadening. Fundamentals of this reactor type have been thoroughly investigated by Hofmann and Halász [26]. An impression of the dependency of peak dispersion, tube length and I.D. is given in Fig. 6. The reduction of the effective plate number N_{eff} of three reactors (250 cm \times 0.3 mm I.D., 500 cm \times 0.5 mm I.D. and 1000 cm \times 0.5 mm I.D.) is plotted over the flow rate of the eluent. To simulate the effect of the fittings and of straight parts of the reactors, capillaries of 0.3 and 0.5 mm I.D. with lengths of 23 and 32 cm were tested at 0.8 ml/min flow rate. The 0.3-mm-I.D. capillary leads to a small $\Delta N_{\text{eff}} = 9.7$ while the effect of the 0.5-mm-I.D. is more pronounced with $\Delta N_{\text{eff}} = 190.7$. Considering the volume of the connection tubes values of 0.6 and 3 for $\Delta N_{\text{eff}}/\mu\text{l}$ were obtained respectively. After subtraction of the contribution of the fittings and straight parts, the peak broadening in the knitted parts of the

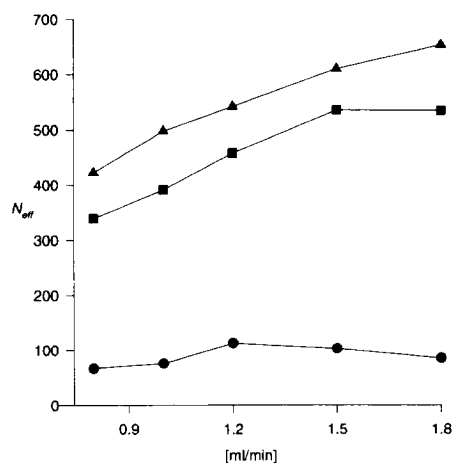


Fig. 6. Contribution of knitted PTFE tubes to peak dispersion. Loss of effective plate number ΔN_{eff} at different flow rates in three reactors: (\blacktriangle) 1000 cm \times 0.5 mm I.D.; (\blacksquare) 500 cm \times 0.5 mm I.D.; and (\bullet) 250 cm \times 0.3 mm I.D. Eluent: 75:25 (v/v) MeCN–H₂O. The variance of the peak distribution was calculated from the peaks of toluene and of the nitrate ion as marker for t_0 .

reactors was calculated: 0.35 $\Delta N_{\text{eff}}/\mu\text{l}$ (0.3 mm I.D., 250 cm total length), 0.16 $\Delta N_{\text{eff}}/\mu\text{l}$ (0.5 mm I.D., 500 cm total length) and 0.12 $\Delta N_{\text{eff}}/\mu\text{l}$ (0.5 mm I.D., 1000 cm total length). The step in I.D. from the column outlet (0.2 mm I.D.) to the reactor tube (0.5 mm I.D.) is the main singular cause for additional band spreading, and introduces the flow dependency into ΔN_{eff} , as can be concluded from the parallel, increasing lines of the 500 and 1000 cm reactor with 0.5 mm I.D. and the low flow dependency of bandspreading in the 250 cm \times 0.3 mm I.D. reactor. This result is in accordance with our qualitative observation of a marked increase in resolution after direct connection of the reactor tube to the fluorescence detector. A 500 cm \times 0.3 mm I.D. knitted tube reactor directly connected to the detector cell thus should induce a $\Delta N_{\text{eff}} \sim 124$. Naturally the effects of the T-piece, the make up of the acid, the increase of viscosity and temperature are not included, however the overall reduction of N_{eff} should be in the order of 1% for a column of 125 mm length, with a 4 μm spherical packing, $N \sim 12\,500$.

3.3. Statistical evaluation

Under these conditions the detection of cardenolides is possible with extreme sensitivity, as shown in Fig. 7. (upper trace) with 0.8 ng/inj. digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside and digoxin corresponding to 2.05, 1.54, 1.23 and 1.03 pmol respectively. The chromatogram of an extracted plasma sample spiked with the same amount of each cardenolide is plotted in the middle trace and the lowest trace represents the corresponding chromatogram from blank plasma. Table 3 summarizes the analytical statistics for the determination with (a) our first post-column derivatization with concentrated H₂SO₄ without addition of Cu(II)acetate, and (b), (c), (d) the results of the final version with 0.08% Cu(II)acetate in H₂SO₄. The day-to-day variations (a) include one course of reassembling the post-column reactor–detector combination and an exchange of the analytical column. Digoxintetradigitoxoside was included in the first series as a potential internal standard, however the variations introduced by peak area integration (data not shown) largely exceeded the variability of the direct evaluation.

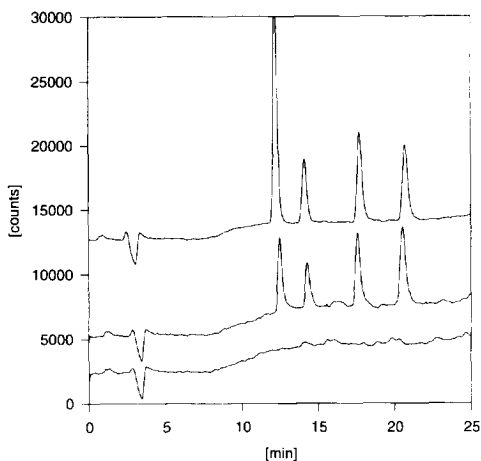


Fig. 7. Chromatographic separation of digoxin, digoxigenin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside with fluorescence detection after post-column derivatization. A 0.8 ng amount of each substance was injected. Retention times: digoxigenin, 12.6 min; digoxigenin monodigitoxoside, 14.5 min; digoxigenin bisdigitoxoside, 18.11 min; digoxin, 21.1 min. Upper trace: standards; middle trace: spiked plasma (1 ml) after SPE; lower trace: blank plasma (1 ml) after SPE.

3.4. Digoxin levels in human plasma samples

In Fig. 8 serum levels of five male healthy volunteers are plotted over 24 h after intake of 0.5 mg digoxin [21]. The increase after 8 h is not statistically significant, but probably corresponds to the effect of intestinal reabsorption of digoxin after excretion via the bile duct. In Fig. 9A data from sixteen elderly, hospitalized patients are shown [22,23] in comparison to values obtained from the same samples with the FPIA. Fig. 9B presents the results for the relation between calculated digoxin equivalents from HPLC and FPIA. No metabolite exceeded 0.49 ng/ml of digoxin equivalents. No correlation between both assays was found. A similar result was obtained from a second group of patients ($n=23$, data not shown here [22]). In a third group serum samples from patients ($n=20$) without digoxin admission were screened for interfering peaks. No disturbing peak was found in this series, or in healthy volunteers, or in commercial plasma samples ($n>15$). None of the investigated steroids from Table 1 interfered with digoxin, nor did spironolacton or furosemid.

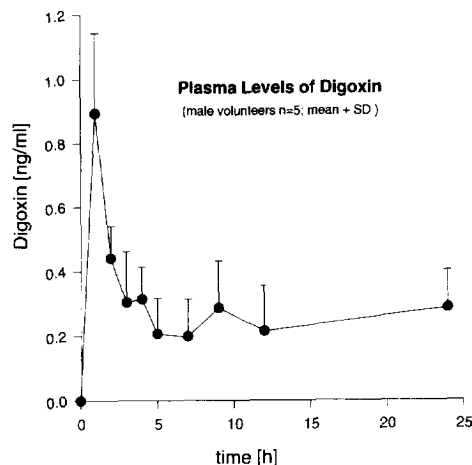


Fig. 8. Digoxin levels in male healthy volunteers after ingestion of one tablet containing 0.5 mg digoxin ($n=5$), monitored over 24 h.

4. Conclusions

The HPLC system combined with post-column derivatization reported in this paper provides a highly sensitive quantification method for cardenolides with a determination limit of ~ 360 fmol/ml and a detection limit of 90 fmol/ml of digoxin in plasma or serum. A closer look at the excellent reproducibility of samples containing 30–40 pg of each cardenolide in Table 3 reveals that potentially even these limits can be lowered, if the calibration range is narrowed or if log–log regression is chosen. Observations about the nature of the derivatization reaction are superficial, because the experiments were directed to method development, but allow the conclusion that the steroid nucleus is the main determinant for the spectral and reactivity characteristics of each compound. Probably one reaction step is elimination of water from the steroid nucleus [17] with participation of hydroxy] 3α . Copper ions are, similar to TLC, remarkable derivatization catalysts and probably part of charge-transfer complexes with the acids. Under optimized conditions the reaction was fast enough to take place in a knitted capillary tube reactor with reaction times of ~ 1 min, which is \sim ten times faster than reaction speeds reported by Gfeller et al. [17] or \sim five times faster than Embree et al. [20] for the derivatization of cardiac glycosides with concentrated hydrochloric

Table 3
Statistical evaluation of the HPLC assay for digoxin and related cardenolides

| Substance | $y = ax + b$ | | r^2 | LOQ (pg) | LOD (pg) | Recovery (%) | S.D. (%) (standards) | S.D. (%) (plasma) ($n = 3$) |
|-----------|--------------|------|--------|-------------|-------------|-----------------|-------------------------|-------------------------------------|
| | a | b | | | | | | |
| D0G (a) | 56.79 | 3925 | 0.9869 | 600 | 200 | 50 | 5.3 (5 days) | 18.5 (4 days) |
| (b) | 66.1 | 7 | 0.9922 | 290 | 80 | 100 | 4.1 (30 pg) | |
| (c) | 30.6 | 2.4 | 0.9730 | 570 | 150 | 46 | | 37.5 (30 pg) |
| (d) | 47.9 | 1.6 | 0.9816 | 510 | 140 | 72 | | 20.9 (30 pg) |
| D1G (a) | | | | | | 68 | | |
| (b) | 82.2 | 9 | 0.9904 | 350 | 90 | 100 | 4.6 (30 pg) | |
| (c) | 73.1 | 1.6 | 0.9788 | 510 | 140 | 89 | | 24.9 (30 pg) |
| (d) | 61.9 | 7.1 | 0.9963 | 280 | 70 | 75 | | 10.0 (30 pg) |
| D2G (a) | 53.79 | 7065 | 0.9972 | 280 | 90 | 83 | 4.1 (5 days) | 5.5 (4 days) |
| (b) | 45.4 | 4.7 | 0.9952 | 290 | 80 | 100 | 16 (40 pg) | |
| (c) | 43.4 | 0 | 0.9952 | 350 | 90 | 96 | | 22.4 (30 pg) |
| (d) | 36.7 | 10.6 | 0.9925 | 400 | 100 | 81 | | 11.9 (40 pg) |
| D3G (a) | 72.33 | 6574 | 0.9982 | 240 | 70 | 95 | 3.6 (5 days) | 6.7 (4 days) |
| (b) | 36.0 | 3.3 | 0.9943 | 230 | 40 | 100 | 8.2 (40 pg) | |
| (c) | 33.5 | 3.2 | 0.9937 | 380 | 100 | 93 | | 15.4 (40 pg) |
| (d) | 28.5 | 2.7 | 0.9958 | 280 | 70 | 81 | | 10.9 (40 pg) |
| D4G (a) | 62.84 | -801 | 0.9904 | 340 | 110 | | | |

Lower limit of detection (LOD) and lower limit of quantification (LOQ) after linear regression according to DIN 32645. Each series consists of three repetitive injections at twelve levels equally spaced over the whole concentration range. (a) Manual SPE of spiked plasma samples with SPE columns on a vacuum manifold, derivatization with 95% H_2SO_4 at 110°C. Range: digoxigenin (D0G) 30–2040 pg per injection, digoxigenin bisdigitoxoside (D2G) 40–2500 pg per injection, digoxin (D3G) 40–2400 pg per injection, digoxigenin tetradigitoxoside (D4G) 200–4000 pg per injection. Day-to-day reproducibility for standards was checked on five consecutive days with two samples (1.09 ng in 33 μ l MeOH; $n = 2$) and plasma samples (1.09 ng/ml; $n = 3$) were analysed on four consecutive days. (b) Standard solutions (300 μ l) without SPE, derivatization with 0.08% (w/v) Cu(II) acetate-95% H_2SO_4 at 75°C. Range: D0G 30–2040 pg per injection, digoxigenin monodigitoxoside (D1G) 30–2200 pg per injection, D2G 40–2500 pg per injection, D3G 40–2400 pg per injection. (c) Aqueous samples, SPE and derivatization as in (a) and (b) respectively. Range: D0G 30–2040 pg per injection, D1G 30–2200 pg per injection, D2G 40–2500 pg per injection, D3G 40–2400 pg per injection. (d) Spiked plasma samples, SPE and derivatization as in (a) and (b) respectively. Range: D0G 30–2040 pg per injection, D1G 30–2200 pg per injection, D2G 40–2500 pg per injection, D3G 40–2400 pg/inj. The S.D. of (b), (c) and (d) was checked for the lowest injected amount on two consecutive days. $n = 3$.

acid–hydrogen peroxide–ascorbic acid mixtures. Dead volume problems were effectively reduced with knitted PTFE-capillaries, which can even be coupled to 2-mm I.D. columns without loss of sensitivity. In contrast, the make up of the column effluent with sulfuric acid should reduce the worsening of peak bandwidth that is normally obtained with the standard 12- μ l flow cell of fluorimeters coupled to narrow bore HPLC-columns with small particle sizes. This may be a valuable starting point for further miniaturization of this system. Differentiation of steroid conjugates [27] from 'true' EDLFs will benefit from the maintained chromatographic performance of the system at considerably increased

sensitivity from 0.5 ng per injection [4] for a non-retained cardenolide to ~0.1 ng per injection during chromatography. Not yet explored is the selectivity enhancement, which can be achieved by recording of fluorescence at different emission wavelengths, which looks promising (Fig. 4 and Fig. 5). The simplicity of the reactor–detector unit may promote the transfer of other TLC derivatization reactions with aggressive media to the HPLC world, i.e. steroids (Table 1) can be detected by the described technique at physiological levels, if the described SPE gets adopted [28]. Corticosteroid determination from human saliva [29] with a similar derivatization medium has been published recently. The extreme

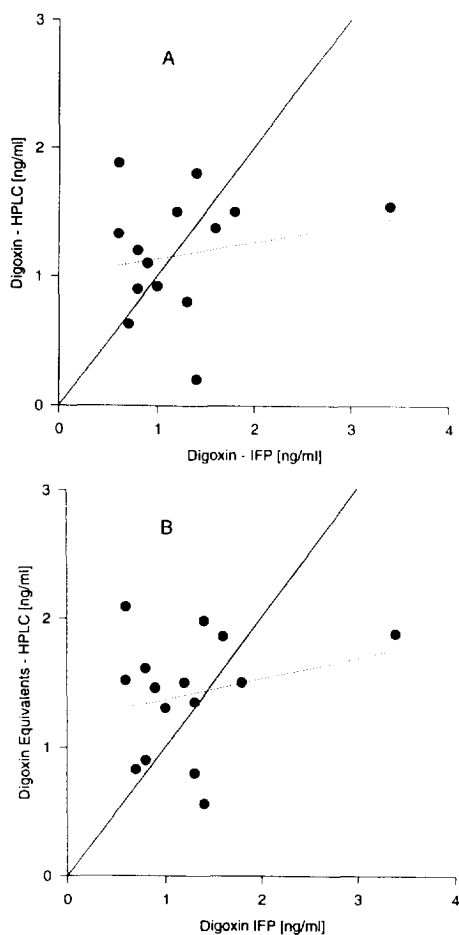


Fig. 9. (A) Digoxin levels in 16 out of 24 elderly patients measured with HPLC and FPIA (TDxII). FPIAs were performed under clinical routine conditions ($r=0.19$). (B) Calculated digoxin equivalents in the samples from A. Digoxin equivalents = Σ ([metabolite] \times crossreactivity) + [digoxin]_{HPLC} over [digoxin]_{FPIA} ($r=0.23$). LLQ of the clinical routine FPIA is 0.5 ng/ml, therefore samples ($n=4$) with less than 0.5 ng/ml in the FPIA were excluded. Five patients with a nonresolved peak near the digoxin peak and were not evaluated. The dashed line represents the ideal response.

stability of the system will be beneficial for routine applications. Typically 10 min after complete reconstruction, the baseline was stable and an injected sample showed less than 10% deviation from previous mean values. The best indicator for the extraordinary power of the SPE method without liquid-liquid extraction steps is the flat baseline obtained after extraction of control plasma samples. Samples for calibration purposes can therefore be directly

Table 4

Results of digoxin determinations in patients under digoxin regimen

| Digoxin FPIA (ng/ml) | Digoxin HPLC (ng/ml) | Digoxin equivalents HPLC (ng/ml) | Classification |
|----------------------|----------------------|----------------------------------|----------------|
| <0.5 | 0.41 | 0.41 | * |
| <0.5 | 0.51 | 0.51 | * |
| <0.5 | 0.35 | 0.52 | * |
| <0.5 | 0.66 | 1.03 | — |
| 0.6 | 1.33 | 1.48 | — |
| 0.6 | 1.88 | 2.15 | — |
| 0.7 | 0.63 | 0.88 | — |
| 0.8 | 0.90 | 0.90 | * |
| 0.8 | 1.20 | 1.60 | * |
| 0.9 | 1.10 | 1.28 | * |
| 1.0 | 0.92 | 1.38 | * |
| 1.2 | 1.50 | 1.50 | * |
| 1.3 | 0.80 | 1.58 | * |
| 1.3 | 0.80 | 0.80 | * |
| 1.4 | 1.80 | 1.97 | * |
| 1.4 | 0.20 | 0.55 | — |
| 1.6 | 1.37 | 1.86 | * |
| 1.8 | 1.50 | 1.50 | * |
| 3.4 | 1.54 | 1.99 | — |

Comparison of FPIA and HPLC and classification into three groups. Group 1, <0.8 ng/ml; group 2, 0.8–2.2 ng/ml; group 3, >2.2 ng/ml digoxin (FPIA) (*, correct classification; —, false classification).

prepared from donor plasma or serum without additional pretreatment to remove endogenous steroids.

Human digoxin levels are amenable for pharmacokinetic investigations and for clinical routine with this HPLC assay without immunological complications. In healthy volunteers, plasma levels exhibit the time curve of a drug with accumulation tendency, documented by the significant plasma levels after 24 h. In patients the most astonishing result was the total lack of correlation between HPLC and FPIA, which is in contrast to other publications comparing HPLC with RIA [30] or FPIA [31]. Possibly the main reason is the enhanced sensitivity and excellent purity of the samples, which prohibits misinterpretations. Positive overestimation of digoxin in patients by the HPLC-assay may be explained by poorly separated metabolite peaks like dihydrodigoxin [20], however all rejected samples ($n=4$ of 24), which exhibited a nonresolved peak at t_{digoxin} , were clearly identified by their doubled peak

width. Alternatively “digoxin” was never detectable in samples from healthy controls or patients without digoxin therapy, and not a single false, negative result was observed with spiked plasma samples from different individuals in HPLC (patients $n=20$; healthy volunteers $n>15$). Our results therefore indicate some unknown factors, which reduce the sensitivity of the FPIA for digoxin. Together with the EDLF complications this displays a strong limitation for the validity of digoxin determination by FPIA. Clinically important is the evaluation in Table 4, because the classification into groups of low digoxin, normal digoxin and critical digoxin by FPIA (group 1, <0.8 ng/ml; group 2, 0.8 – 2.2 ng/ml; group 3, >2.2 ng/ml) failed in 31% of all cases ($n=19$).

A way out of these problems encountered by FPIA of digoxin may be sample pretreatment according to the SPE sequence described above, to eliminate bulk contaminations. However such an attempt has to be investigated thoroughly and cannot be as reliable as a complete chromatographic determination, which allows the identification of critical samples (individuals).

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