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Bioanalysis of digoxin and its metabolites using direct serum injection combined with liquid chromatography and on-line immunochemical detection

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Abstract

An automated dual-column liquid chromatographic assay for digoxin is described. Serum samples are directly injected onto a restricted-access solid-phase extraction support. After liquid chromatographic (LC) separation on a C_{18} analytical column, antigenic analytes are detected by means of post-column immunochemical detection (ICD) using fluorescein-labelled antibodies against digoxigenin. The detection limit of this assay is 160 pg/ml (preconcentration of 1.0 ml serum). With the present method digoxin and three of its cross-reactive metabolites were determined in serum taken from patients which were orally administered a 1-mg dose of digoxin. The results obtained with LC–ICD were compared with data provided by a batch immunoassay.

1. Introduction

Heart-glycosides, *e.g.* digoxin, are still widely used in treatment of heart failure and arrythmias. Due to the low therapeutic index of digoxin, dosing is important, therefore accurate measurement of digoxin concentrations is required. One of the major problems in the measurement of digoxin in human serum is that clinical concentrations (0.5–2.0 ng/ml) are too low for conventional HPLC analysis whereas simple immunological assays, which are very sensitive, suffer from cross-reacting substances, *e.g.* metabolites, co-administered drugs and digoxin-like immunoreactive substances (*e.g.* cortisol and progesterone) [1].

A large number of different immunoassays were developed for digoxin and its metabolites. A flow-injection enzyme immunoassay was described by Freytag *et al.* [2] based on the off-line incubation of the sample with enzyme-labelled antibodies. In a continuous-flow system, free and bound antibodies are separated using a short affinity column packed with an immobilized-antigen support. After addition of substrate via a mixing union the amount of bound antibody was determined by colorimetric detection. A similar detection technique was recently reported by Gunaratna and Wilson [3] for α-(difluoromethyl)ornithine.

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Analytical methods which can distinguish between digoxin and its cross-reactive metabolites are mainly based on HPLC separations. A sensitive post-column reaction–detection system in combination with fluorescence detection has been reported providing a detection limit of 0.5 ng [4]. To enhance selectivity, immunopreconcentration techniques were applied for the isolation of digoxin from biological matrices [5]. HPLC fractionation has been used prior to immunoassays to eliminate interfering (cross-reactive) substances [6].

In recent years the implementation of immunoaffinity techniques in liquid chromatography or capillary electrophoresis has become more and more popular. A review on the efforts undertaken so far was recently published by De Frutos and Regnier [7]. Our group reported the on-line coupling of immunoassays with liquid chromatographic (LC) separations with the goal to overcome the need of tedious fraction collection [8]. We showed that immunochemical reactions are compatible with reversed-phase LC separations by performing a reaction sequence based exclusively on the fast association of antibodies with antigens. After post-column addition of fluorescein-labelled antibodies against digoxigenin, free and antigen-bound antibodies were separated by means of an immobilized-antigen affinity column. The antigen bound fraction then was detected using fluorescence detection.

In the present paper the application of this immunochemical detection (ICD) method to the bioanalysis of digoxin and its metabolites in serum is described. Due to the high selectivity of the detection method, sample pretreatment was limited to unselective on-line solid-phase extraction (SPE) using restricted-access supports based either on silica [9] or polystyrene [10,11]. Both supports allowed the repeated direct injection of plasma or serum samples. The present method enables the determination of digoxin and three of its cross-reactive metabolites in serum taken from patients which were administered orally with a 1-mg dose of digoxin. The results obtained with LC–ICD were compared with data provided by a batch fluoroimmunoassay.

2. Experimental

2.1. Chemicals and supports

Digoxin was obtained from Janssen Chimica (Geel, Belgium). Bovine serum albumine (BSA), digoxigenin, digoxigenin monodigitoxose, digoxigenin didigitoxose, dihydrodigoxin, spironolactone and dehydroepiandrosterone-3-sulphate were purchased from Sigma (St. Louis, MO, USA). Tween 20 was obtained from Merck (Darmstadt, Germany). Acetonitrile was obtained from Rathburn (Walkerburn, UK). All other organic solvents were purchased from J.T. Baker (Deventer, Netherlands) and were of analytical grade. Cortisol and progesterone were obtained from Akzo Organon (Oss, Netherlands). Furosemide was obtained from Aldrich (Milwaukee, IL, USA). Affinity-purified fluorescein-labelled Fab fragments of polyclonal anti-digoxigenin (Fab-DIG) were obtained from Boehringer Mannheim (Mannheim, Germany). Styrosorb precolumns were a gift of Prof. Davankov (University of Moscow, Moscow, Russian Federation). C_{18} alkyl-diol silica precolumns were donated by Prof. Boos (University of Munich, Munich, Germany) [9].

2.2. HPLC system

The principle of post-column ICD has been described in ref. [8]. All experiments were carried out using an LC system consisting of two Kratos-ABI (Ramsey, NJ, USA) Spectroflow 400 pumps, a Gilson (Villiers-le-Bel, France) 231 autosampler, a MUST (Spark Holland, Emmen, Netherlands) multiport streamswitch, a 125 × 4.6 mm I.D. LiChroCART C_{18} column, particle size 5 μm (Merck, Darmstadt, Germany), a Pharmacia (Uppsala, Sweden) P3500 pump for post-column addition of antibodies and a Perkin-Elmer (Beaconsfield, UK) LS-4 fluorescence detector (excitation wavelength 480 nm, emission wavelength 514 nm). The mobile phase for the precolumn consisted of 0.2 mol/l sodium acetate (pH 7.0) pumped at a flow-rate of 1.0 ml/min. The LC mobile phase was acetonitrile–0.05 mol/

l sodium acetate pH 7.0 (30:70, v/v) pumped at a flow-rate of 0.50 ml/min. The immunoreagent solution consisted of phosphate-buffered saline (PBS) containing 1.3 nmol/l Fab-DIG and 0.5% Tween 20 and was used at a flow-rate of 1.0 ml/min. Mixing of the eluent with the immunoreagent solution was performed by using an inverted Y-type low-dead-volume mixing union. The reaction coil (volume, 1600 μl) consisted of 0.5-mm I.D. knitted PTFE tubing; the reaction was performed at 20°C. A 10 × 4.0 mm I.D. column, slurry packed with Carbolink-hydrazide (Pierce, Rockford, IL, USA) coupled digoxin was used to bind free antibodies. A scheme of the analytical system is displayed in Fig. 1.

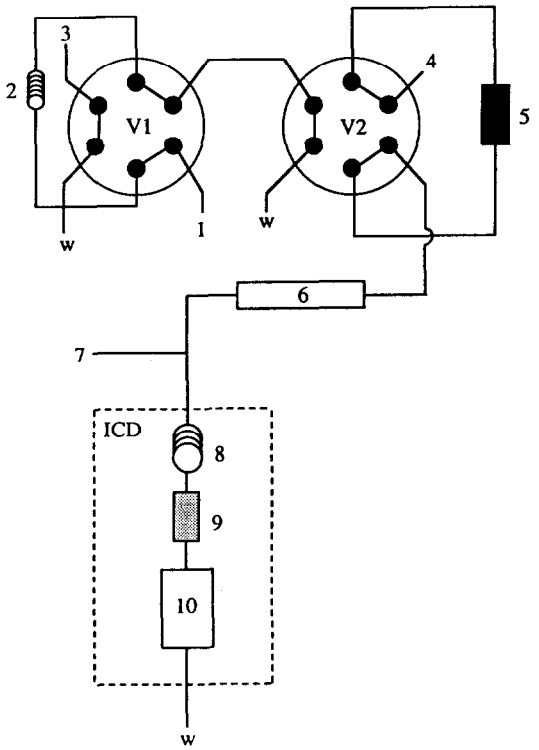

Fig. 1. Scheme of the analytical system. (1) Preconcentration pump, (2) sample injection loop (1000 μl), (3) autoinjector, (4) HPLC pump, (5) pre-column packed with restricted-access support, (6) analytical column, (7) reagent pump, (8) reaction coil, (9) affinity column packed with immobilized-antigen support, (10) fluorescence detector, (V1,V2) six-port injection valves, (ICD) immunochemical detection system, (w) waste. Conditions, see Experimental.

The analytical system was controlled by a Gilson 605 GSIOC driver software connected to a personal computer.

2.3. Sample handling and standard solutions

Blood samples were taken in 5-ml plain tubes (Sarstedt monovette) and left to clot. Serum was separated and stored at −40°C until required for analysis. Before analysis samples were thawed, 5% acetonitrile was added and the sample was centrifuged for 10 min at 5200 *g*. Prior to injection of the sample the C_{18}-alkyl diol restricted-access precolumn was equilibrated with 3 ml of preconcentration buffer. One ml supernatant was then directly injected onto the precolumn and high-molecular mass components were removed by washing with 18 ml of preconcentration buffer. By switching valve 2, compounds adsorbed on the C_{18}-bonded silica were desorbed on-line to the analytical column.

A stock solution of digoxin and its metabolites was made in acetonitrile (100 μg/ml). This solution was diluted to give solutions of 2 to 200 ng/ml in acetonitrile. Calibration curves were performed by adding 250 μl of digoxin solutions to 4750 μl blank plasma. After centrifugation for 10 min at 5000 *g*, 1 ml of the supernatant was injected.

2.4. Batch immunoassay

The determination of digoxin by means of a polarization enzyme immunoassay (TDX Digoxin II) was performed using a standard automatic analyser (TDX, Abbott Laboratories, Diagnostics Division, North Chicago, IL, USA).

3. Results and discussion

3.1. Influence of cross-reactive compounds

In batch immunoassays the signal obtained reflects the sum of the total concentrations of all cross-reactive compounds present in the sample. It can be expected that the same compounds can

also be detected if the immunochemical detection technique is coupled on-line to a liquid chromatographic separation system. Theoretically, compounds having a binding constant similar to digoxin should be detected with the same sensitivity. The response of compounds with a weaker affinity for anti-digoxigenin could be lower if the immunocomplex formed in the first immunoreaction reacts with the immobilized digoxin. This reaction would remove antibodies bound already to the analyte and therefore result in a lower response factor.

Anti-digoxigenin antibodies are known to cross-react with other steroid-like compounds such as cortisol or progesterone although the binding constants of the resulting immunocomplexes are considerably lower [12]. Therefore the response factors of digoxin, its metabolites and a number of potentially cross-reactive compounds were determined by flow-injection analysis (FIA; for results see Table 1). It was found that all compounds possessing the intact digoxigenin moiety provided virtually identical response factors. Dihydrodigoxin, where the double-bond at the card-20(22)-enolide ring is reduced, has a five-times lower response factor than digoxin. Other steroid-like compounds such as cortisol or progesteron were not detected at all. Apparently an intact C- and D-ring system of digoxigenin is an essential requirement for compounds to be detected with anti-digoxigenin antibodies.

Table 1
Response factors (RF) and capacity factors (k') of cross-reactive compounds

Compound	RF[a]	k'[b]
Digoxigenin	100	0.75
Digoxigenin monodigitoxose	100	0.85
Digoxigenin didigitoxose	100	1.05
Digoxin	100	1.43
Dihydrodigoxin	20	1.43
Spironolactone	< 0.1	N.D.[c]
Progesterone	< 0.1	N.D.
Dehydroepiandrosterone-3-sulfate	< 0.1	N.D.

[a] Values measured by means of FIA–ICD. Response factors were calculated by dividing the peak area by the injected amount of analyte and normalized with respect to the response factor obtained for digoxin ($R_{digoxin} = 100$).
[b] Values measured by means of LC–ICD.
[c] N.D. = not determined.

3.2. Sample pretreatment using restricted-access supports

By implementing a highly selective detection technique based on antibody–antigen interactions sample handling can be restricted to the efficient and fast isolation of the analytes from complex matrices such as plasma, serum or urine. Solid-phase extraction using restricted-access supports which allow the repeated direct injection of untreated plasma samples was chosen for this purpose. Two different restricted-access materials were investigated, C_{18}-alkyl diol modified silica [9] and a Tris-modified polystyrene support, Styrosorb [10]. Both stationary phases are composed of a hydrophilic outer layer and hydrophobic pores. They differ in hydrophobicity and pore size.

The recoveries of digoxin and its metabolites both from aqueous solutions and from serum samples (1 ml injected directly onto the supports) were higher than 90% for both supports. On-line desorption of digoxin with the LC mobile phase containing 30% acetonitrile provided good peak shapes if operated in the back-flush mode. In the forward-flush mode, the size-exclusion properties of both supports provided rather broad peaks. Due to the larger hydrophobicity of the polystyrene support compared to that of the C_{18} analytical column, peak broadening after desorption was larger than that of the C_{18}-alkyl diol support. The characteristics of the Styrosorb support and its use in bioanalysis will be described elsewhere [13]. The bioanalytical method for the determination of digoxin and its metabolites was optimized using C_{18}-alkyl diol silica as restricted-access support.

After injection of 1 ml serum onto the support proteins were removed by flushing with 18 ml of acetate buffer (0.2 mol/l, pH 7.0) or water. In this way one hundred injections of 1.0 ml serum could be performed before backpressure built up and an increase of peak broadening was ob-

served. The problem of increased backpressure was probably caused by clogged frits and could be solved by cleaning the precolumn regularly with 250 ml acetonitrile–water (40:60, v/v).

3.3. Bioanalysis of digoxin and metabolites

Determination of digoxin, digoxigenin didigitoxose, digoxigenin monodigitoxose, digoxigenin and dihydrodigoxin in serum was carried out using direct serum injection on C_{18}-alkyl diol bonded silica, separation on a C_{18}-bonded silica packed analytical column and detection using the immunochemical detection method. For 1.0-ml injections of serum a detection limit (signal-to-noise ratio = 3) for digoxin and its metabolites of 0.2 nmol/l was obtained which is below the range of the clinical effective concentrations (0.5–5 nmol/l). The method was validated by the fivefold assay on five different days of blank plasma samples to which different amounts of digoxin were added. Data for reproducibility and accuracy are presented in Table 2. The day-to-day reproducibility is in the same range as the within-day variability. The detector response was linear ($r = 0.995$, $n = 5$) between 0.2 and 2 nmol/l. The narrow range of linearity can be attributed to the low antibody concentration (1.3 nmol/l) in the reagent solution. By applying non-linear curve fitting the upper limit of the calibration curve could be extended to 10 nmol/l at a constant correlation coefficient.

The reactivity of the antibodies differed from batch to batch resulting in peak height variations of 30% for different batches. This variation derives from the heterogeneous nature of the polyclonal antibodies used. Probably, the use of monoclonal antibodies which have a well defined reactivity and specificity would lead to a reduction of this variability.

Table 2
Precision and accuracy ($n = 5$) for the determination of digoxin in plasma using LC–ICD

Concentration (n*M*)	Precision (%)		Accuracy (%)
	Within-day	Between-day	
0.2	10.0	11.8	118.3
0.5	5.3	6.7	98.8
1.0	7.5	9.8	103.1

A chromatogram representing the analysis of blank serum and serum obtained after oral administration of 1 mg digoxin is shown in Fig. 2. With the present chromatographic system, digoxin, digoxigenin didigitoxose, digoxigenin monodigitoxose, and digoxigenin were separated. Digoxin and the pharmacologically inactive dihydrodigoxin were not resolved. No attempts were undertaken to separate these two compounds, *e.g.* by employing chloroform in the mobile phase [4]. However, it is expected that the error due to dihydrodigoxin is negligible since the response factor of dihydrodigoxin at concentrations of 0.1 to 10 nmol/l is fivefold lower than that of digoxin and the mean amount of formed dihydrodigoxin by metabolism is 10–15%, although there are cases reported of metabolism up to 50% [14].

3.4. Comparison of immunochemical detection with batch immunoassay

On-line coupling of immunochemical detection with liquid chromatography should provide more

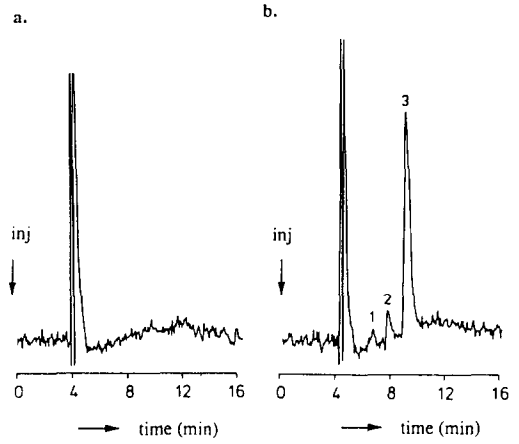

Fig. 2. Determination of digoxin and its metabolites in serum using LC–ICD. Conditions: see Experimental. (a) Blank serum, (b) patient serum after oral administration of 1 mg digoxin. Peaks: (1) digoxigenin monodigitoxose, (2) digoxigenin didigitoxose, (3) digoxin. The digoxin peak has a concentration of 2.7 nmol/l.

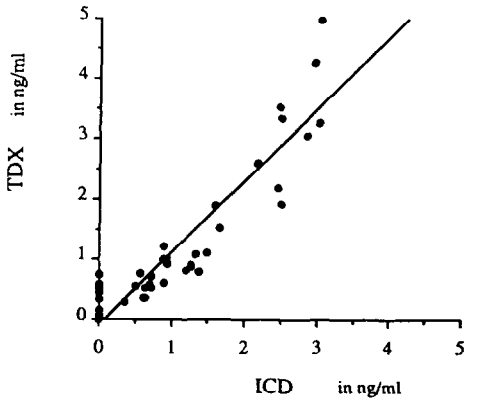

Fig. 3. Correlation of digoxin concentrations measured in patient serum by TDX immunoassay (x-axis) and LC–ICD (y-axis).

accurate data compared to conventional batch immunoassays since quantitation of the majority of cross-reactive compounds is possible. The presence of the cross-reactive metabolites digoxigenin didigitoxose and digoxigenin monodigitoxose was detected with LC–ICD suggesting that digoxin concentrations measured by the immunoassay should be higher than those provided by the LC–ICD technique. The present analytical method was therefore used to re-analyze clinical samples having been analyzed previously by means of a fluoroimmunoassay. In Fig. 3, the concentrations of digoxin found with LC–ICD are plotted against the concentrations obtained by the TDX assay. The correlation of these data is reasonable ($r^2 = 0.91$, $n = 45$). The TDX data are approximately 20% higher than the values obtained by LC–ICD over the whole concentration range measured. This can partly be explained by the fact that in the batch immunoassay the sum of all cross-reactive compounds is determined while the LC–ICD technique is capable to distinguish between digoxin and its cross-reactive metabolites. Fig. 4 presents the plasma concentration–time profile of digoxin after a bolus injection of 1 mg.

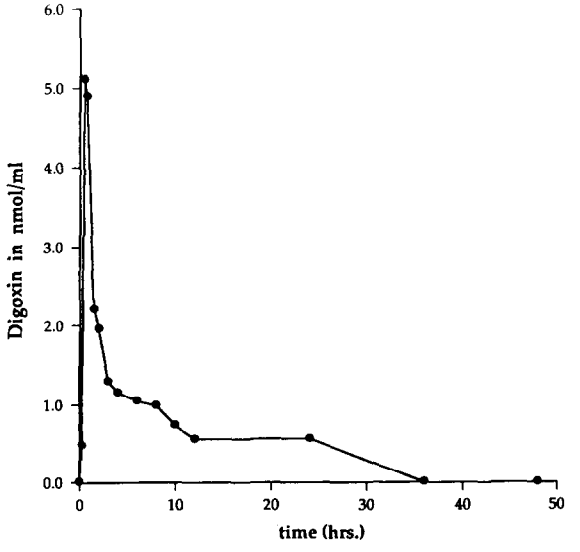

Fig. 4. Plasma concentration–time profile of digoxin after a bolus injection of 1 mg.

4. Conclusions

Immunochemical detection coupled on-line to liquid chromatography is capable to determine cross-reactive analytes at sub-nmol/l concentrations without requiring fraction collection including the performance of immunoassays for the individual fractions. The high selectivity of the method permits a rather unselective sample pretreatment technique – on-line solid-phase extraction on C_{18}-bonded silica or polystyrene/divinylbenzene copolymers – while providing sensitivities similar to batch fluoroimmunoassays. By using restricted-access supports which allow direct plasma or serum injection, the only off-line sample pretreatment step is centrifugation to remove particulate matter.

Compared to non-biochemical detection techniques a somewhat lower reproducibility and range of linearity is observed. In both cases the nature of the reagent – fluorescein-labelled antibodies – is responsible for these shortcomings. The lower reproducibility is mainly caused by batch-to-batch differences among different antibody preparations. The small range of

linearity can be attributed to the low antibody concentration (1.3 nmol/l) used. An increase in the antibody concentration would result in an increase in noise caused by the non-binding fraction (approx. 35% of the total antibody concentration). However, despite these restrictions on-line ICD is advantageous to batch immunoassays, if accurate data on the individual antigens is required. Furthermore, this method is suitable for detection of yet unknown antigenic compounds at high sensitivity. Cross-reactivity studies demonstrated that only highly cross-reactive antigens are detectable with ICD providing a degree of identification which is only matched by LC–mass spectrometry.

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