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DETERMINATION OF DIGOXIN IN SERUM BY ON-LINE IMMUNOADSORPTIVE CLEAN-UP HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND FLUORESCENCE-REACTION DETECTION

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SUMMARY

The use of valve-switching with an anti-digoxin immunoadsorptive clean-up cartridge prior to reversed-phase elution of serum is described. Eluted digoxin is detected by reaction of the unsplit eluent stream with hydrochloric acid in a PTFE coil, followed by fluorescence detection. Routes for optimization and evaluation of the immunoadsorptive clean-up are reported, and the effects on the recovery rate examined The detection limit of 300 pg/ml and reproducibility of less than 7% for the application of the analytical procedure to serum are reasonably good.

INTRODUCTION

Determination of digoxin in serum has long been a domain of immunological assays. The first gas chromatographic method, proposed by Watson and co-workers [1–3], consumed large amounts of time and sample, and demanded a highly sophisticated clean-up.

A fully automated high-performance liquid chromatographic (HPLC) method was developed for analysis of digoxin in serum [4,5] by combining simple reversed-phase adsorption clean-up, reversed-phase separation and fluorescence-reaction detection. This method was shown to be useful for confirmation of immunological results by comparison of various assays [6]. In addition to the improvement due to reaction detection (digoxin reacts with hydrogen peroxide–hydrochloric acid in an unsegmented "knitted" PTFE coil to afford a fluorescent derivative within 5 min), an important feature of the method is the elimination of manual sample preparation by incorporation of an RP-2 injection column to adsorb the serum components. This is especially significant since low concentrations of digoxin (0.5–2 ng/ml) are very susceptible to losses and poor reprodu-

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cibility when manual clean-up is performed. Analysis of many serum samples has shown that nearly all chromatograms exhibit well separated digoxin peaks, which can be precisely integrated. In some cases, however, the chromatogram shows peaks of unidentified components, making it difficult to isolate the relevant digoxin peak.

During evaluation of the above-mentioned method, it became apparent that determination not only of digoxin but also of metabolic compounds is necessary. This is not possible during a single run. Furthermore, in parallel development work on a method for digitoxin, it was determined that the recovery rate of this drug is excessively low due to the high level of protein binding (more than 90%). Thus, the objective of this study was to develop a chromatographic method that allows the determination of digoxin (and analogues such as methyl- or acetyldigoxin), digitoxin and their metabolites in a single run. The clean-up step must be improved to afford a chromatogram that is essentially free of contamination peaks, allowing gradient elution of all analytes of interest.

To improve the accuracy of the determination, some investigators [7–11] have combined the separation efficiency of HPLC with the selectivity of immunoassays by fractionating the HPLC eluate and determining the content using off-line immunological methods.

Replacing the reversed-phase packing in the suggested RP-2 injection column with an anti-digoxin immunoadsorptive material (Silica-PAB⟨anti-digoxin⟩IS) would be expected to result in selective sorption of digoxin and related compounds prior to HPLC separation. During the course of this work, Johansson [12] published an adaptation of this principle to the determination of phenytoin in serum. The detailed work presented here confirms that this clean-up method is generally useful for determination of compounds in difficult matrices.

EXPERIMENTAL

Chromatographic system

Chromatograph 1, HP 1090 with autosampler (Hewlett-Packard, Palo Alto, CA, U.S.A.); data system, Maxima 780 (Waters, Milford, MA, U.S.A.); immunoadsorption cartridge, PAB⟨anti-digoxin⟩IS bound to Spherosil, 10 mm×4.6 mm I.D. [13]; separation column, Shandon Hypersil ODS, 50 mm×4.6 mm I.D., 3 μm (Shandon, Astmor, U.K.); precolumn, Synchropak RP4, WP, 5 mm×4.6 mm I.D., 5 μm (Synchrom, Linden, IN, U.S.A.); eluents, (A) 40 μl hydrogen peroxide+5 ml water (double distilled), (B) 250 μl A+80 μl phosphoric acid+200 ml water (double distilled), (C) 2% (v/v) hydrochloric acid in water (25%, v/v), (D) methanol; B–C–D, 20:35:45; flow-rate, 1 ml/min, isocratic; chromatograph 2, Gynkothek 300 B (Gynkothek, Munich, F.R.G.); eluent, 10 m*M* aqueous phosphate buffer, pH 6.8; flow-rate, 0.5 ml/min.

Reaction detector

This was used as described elsewhere [5].

Reagents and solutions

Methanol (Uvasol grade), hydrogen peroxide (Uvasol grade), 30% (v/v), hydrochloric acid, p.a., 37%, hydrochloric acid (Uvasol grade), 25%, phosphoric acid, p.a., 85%, potassium dihydrogenphosphate p.a., Tween 20 and hexane, p.a., were all supplied by E. Merck (Darmstadt, F.R.G.)

Precinorm U, Precinorm L, Precilip, Precilip E.L., Precipath U, affinity adsorbent (100–200 μm, 300 nm, 10 m^2/g), polyclonal antibody〈anti-digoxin〉 immunoadsorptive prepared, polyclonal antibody〈anti-triiodothyronine〉 immunoadsorptive prepared, control sera, antisera and silica adsorber were all supplied by Boehringer-Mannheim (Mannheim, F.R.G.). Digoxin (Serva, Heidelberg, F.R.G.) and bovine serum albumin (BSA) (Behring, Stuttgart, F.R.G.) were also required.

RESULTS AND DISCUSSION

Analysis of pure analytes: development of the method

The desired selectivity is obtained by using an immunoadsorber instead of the RP-2 clean-up cartridge. The arrangement and consequent valve-switching module is shown in Fig. 1.

The sorbent contains a polyclonal antibody against digoxin covalently linked to Spherosil (Silica-PAB〈anti-digoxin〉IS) [13]. It is generally accepted that immobilized proteins (the antibody in this case) are often more stable than proteins in solution. Thus, it is possible to sorb digoxin repeatedly to the adsorber and desorb it under denaturating conditions. Sorption, purging and desorption should be precisely monitored to confirm the recovery rate in this clean-up step when a new sorbent is used.

Adsorption of digoxin. It is very simple to produce adsorption onto an immunoadsorber. Injection of an appropriate volume of serum by the autosampler (400 μl in this case) instantly fixes the digoxin to the adsorber. The valve setting is illustrated in Fig. 1a. The injection column is kept clear by flushing with 1 ml of phosphate puffer (pH 6.8, 10 m*M*) injected separately or introduced by another pump (for removal of proteins, lipids, etc.) prior to purging (valve setting 1a).

Reduction of non-specific sorption. Purging with solutions of methanol or Tween 20 can reduce interferences due to non-specifically bound compounds. It was sufficient and appropriate to use 0.5-ml portions of the solution (injected by the autosampler) for purging. The valve remains in position 1a.

Purging with methanol. Fig. 2 illustrates the effect of the methanol concentration in the purging solution. Denaturation of the antibodies is enhanced, and bound digoxin is slightly displaced by increasing the methanol concentration.

Purging with Tween 20. As in purging with methanolic solutions, increased concentrations of the additive (in this case Tween 20) in the purging solution (0.5 ml) weaken binding of digoxin to the antibody and reduce the recovery rate (Fig. 3). It is thus possible, if necessary, to employ a 10–25% aqueous solution of methanol or a 0.1–0.25% aqueous solution of Tween 20, or a combination of these, to avoid non-specific binding of unknown compounds to the adsorber.

Desorption with hydrochloric acid. Desorption of digoxin from the adsorber

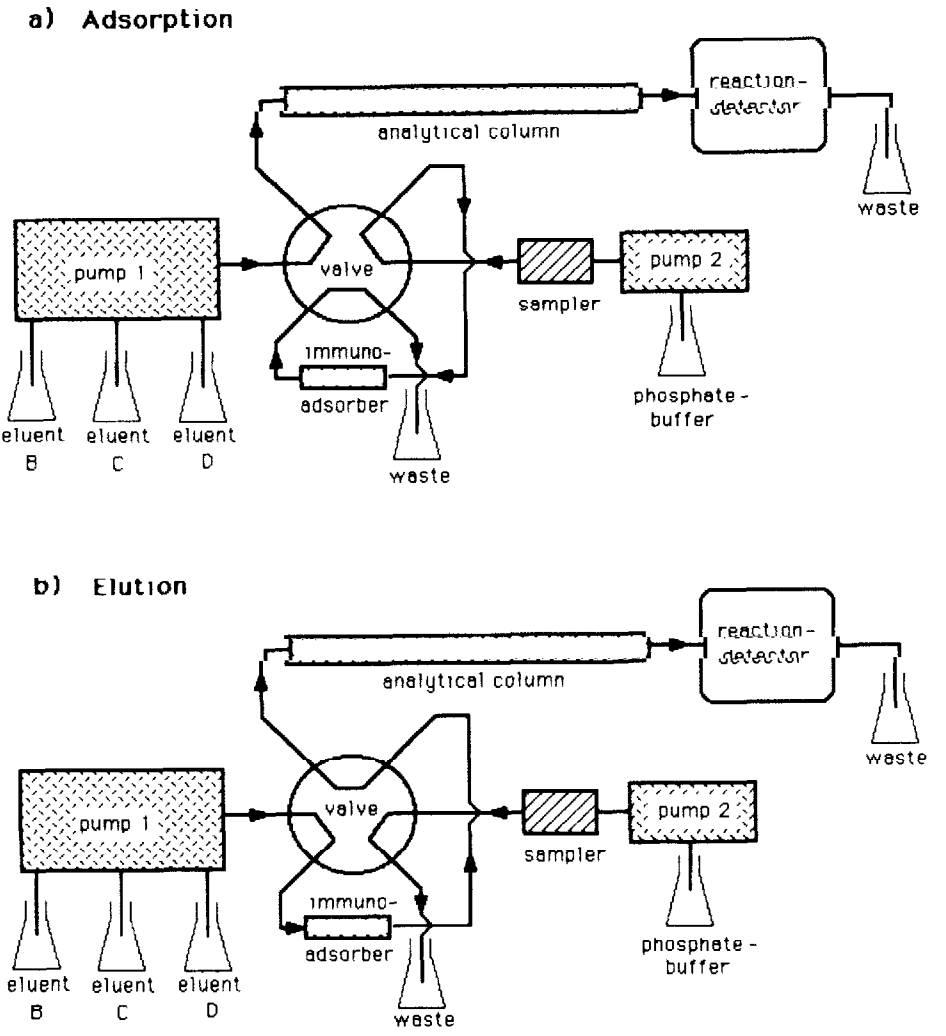

Fig 1. Arrangement of the valve-switching module with clean-up cartridge and reversed-phase column: (a) valve setting for solute sorption; (b) valve setting for elution.

requires harsh conditions. A 1% solution of hydrochloric acid in the eluent reversibly disrupts the secondary structure of the protein in the reaction detector and frees digoxin (Fig. 1b). No significant band-broadening was observed when the liberated digoxin was back-flushed from the adsorber into the separation column with the eluent. Fig. 4 shows that an acid concentration of at least 0.5% hydrochloric acid is necessary to obtain complete recovery, 1% hydrochloric acid solutions being optimal. Higher concentrations should be avoided, since increased destruction of reversed-phase bonding in the analytical column is possible.

Analysis of pure analytes: characterization of the method

Linearity. Calibration was carried out by injecting 0.5, 1.0, 2.0 and 4.0 ng of digoxin. The resultant curve was a straight line through the origin within the limits of confidence. The calibration function was $y=1700.9x+5.27$, where

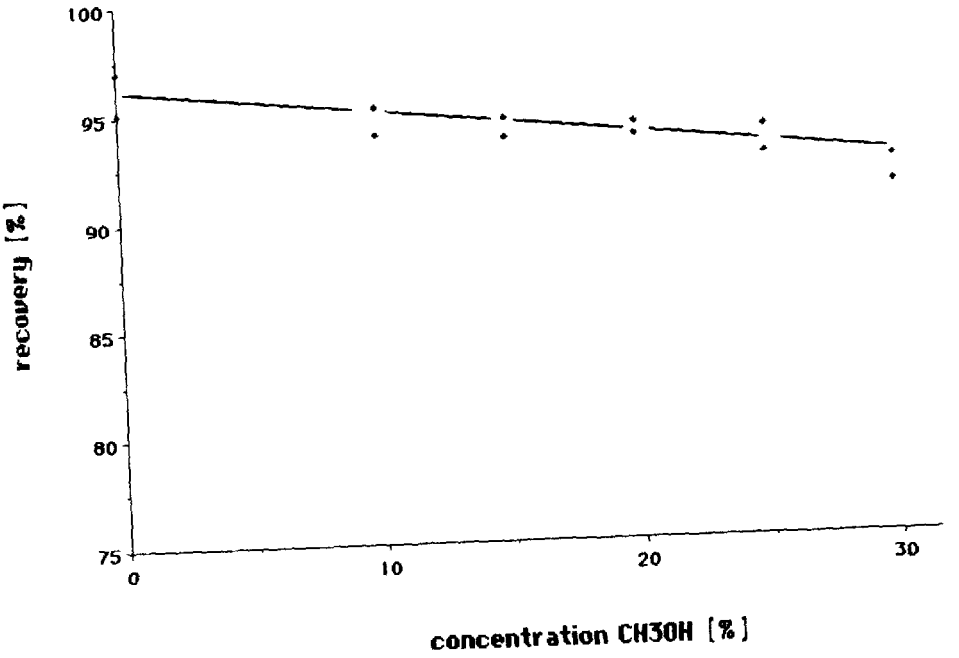

Fig. 2. Effect of methanol purging on solute recovery.

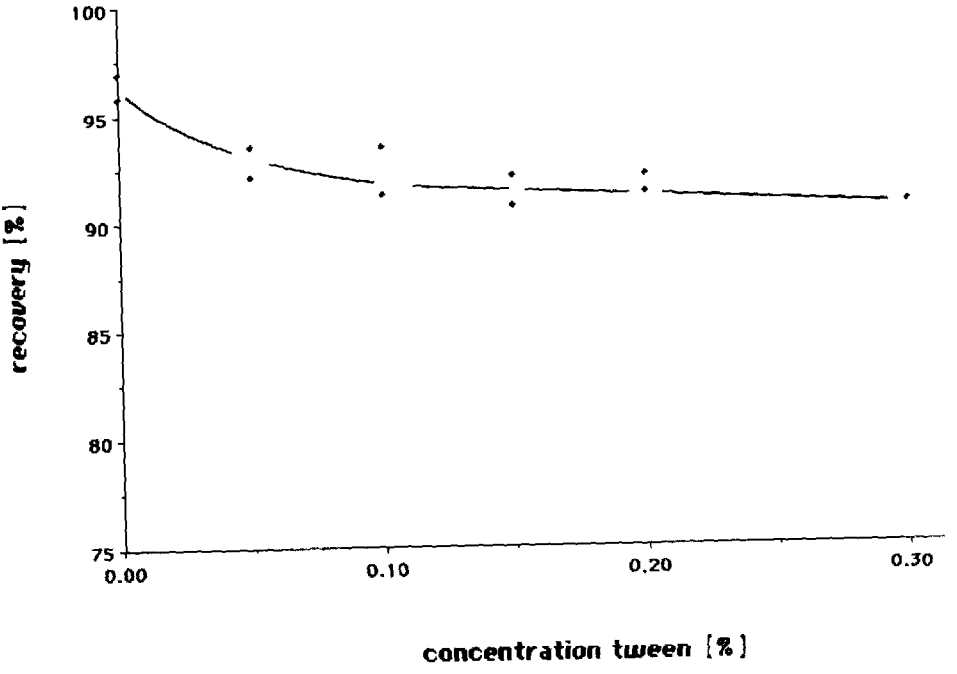

Fig. 3. Effect of Tween purging on solute recovery.

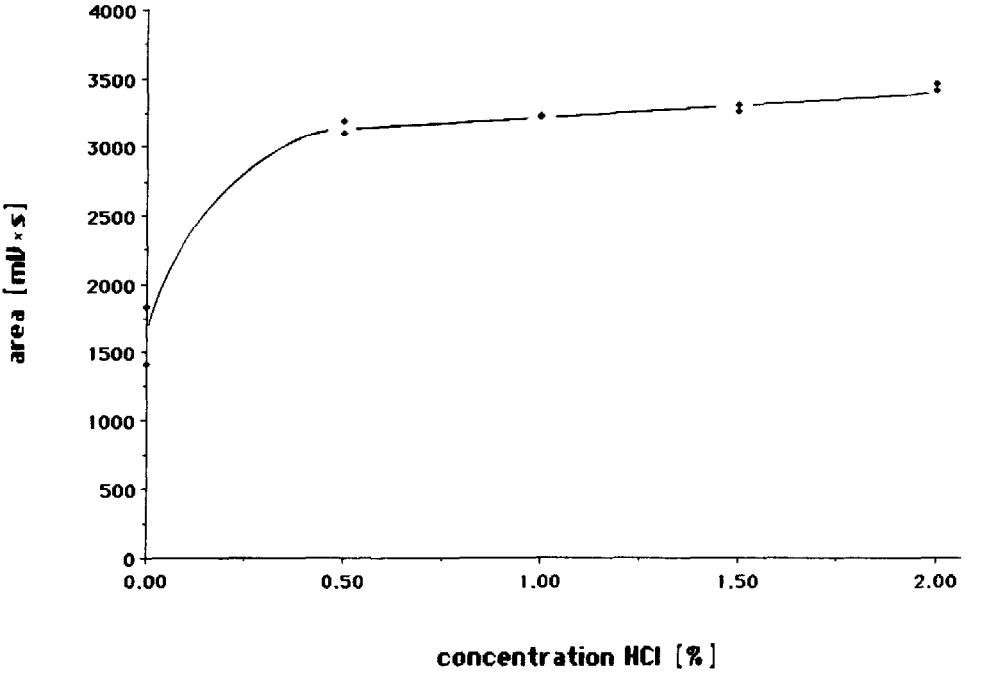

Fig. 4. Variation of solute desorption from the immunoadsorber with different hydrochloric acid concentrations in the eluent.

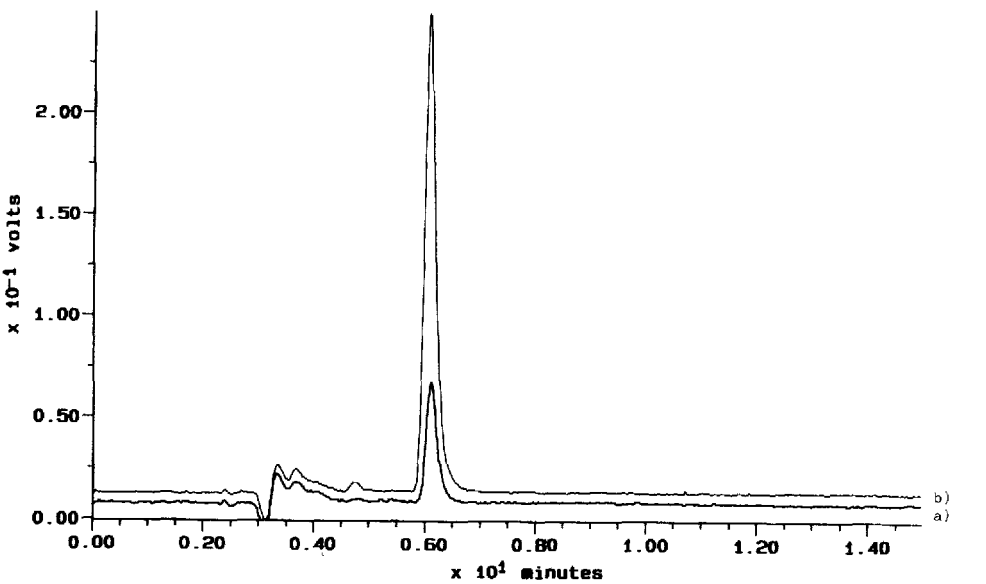

Fig. 5. Elution of aqueous standards: (a) 0.5 ng digoxin per injection; (b) 2.0 ng digoxin per injection.

$r^2 = 0.99994$ (area y in mV s; concentration x in ng per injection). Chromatograms of 0.5 and 2.0 ng digoxin per injection are illustrated in Fig. 5.

Detection limit. The detection limit, at a signal-to-noise ratio of 3:1 may be

TABLE I

EFFECT OF PURGING ON NON-SPECIFIC BINDING OF DIGOXIN TO AN ANTI-TRIIODOTHYRONINE IMMUNOADSORBER

Recovery rate (%)	Purging
15.2	No purging
0	0.5 ml of 25% aqueous methanol
0	0.5 ml of 0.25% aqueous Tween 20
0	0.5 ml of 25% aqueous methanol plus 0.5 ml of 0.25% aqueous Tween 20

calculated to be nearly 50 pg per injection, based on the chromatogram shown in Fig. 5a.

Reproducibility. Pure analytes were eluted at increased eluent strength (55% methanol) to obtain short analysis times. The ratio of the peak height to noise was very favourable, resulting in good reproducibility. The relative standard deviations (R.S.D.) are 1.6% for the peak area and 1.3% for the peak height ($n=12$, 2 ng digoxin per injection).

Recovery. The overall recovery rate was determined by comparing a direct injection of 2 ng of digoxin onto the analytical column (100 ng/ml, 20 μl) with an injection onto the immunoadsorber (2 ng/ml, 400 μl) followed by desorption with the acidic eluent. The recovery rate of 98.5% is quite good for these circumstances.

Selectivity. The selectivity of this type of clean-up procedure primarily depends on the cross-reactivity of the bound antibody. In addition, non-specific binding of foreign compounds can take place. Digoxin binding to an anti-triiodothyronine immunoadsorber was measured to evaluate the non-specificity. Ideally, no binding of digoxin to this adsorber should occur and the recovery rate should be zero. Table I gives the results.

Stability. Repeated sorption and desorption may affect the immunoadsorber by destroying the bound antibodies. No decrease in the digoxin recovery rate was observed when the same sample was injected again and again over a 24-h period. Slight shifts within this series exist (perhaps resulting from instabilities of the reaction detector), but the reproducibility of all measurements was below 7% ($n=40$). It can be assumed that injection of large serum samples (0.4 ml per injection) may reduce the long-term stability.

Application to serum samples: characterization of the method

The following results were determined without purging.

Linearity. Fig. 6a and b show the calibration functions for the peak area and peak height. Both of these are straight lines through the origin (within the limits of confidence): $y=319.81x-26.03$, where $r^2=0.994$ (area y in mV s; concentration x in ng per injection); $y=10.65x-0.266$, where $r^2=0.996$ (height y in mV; concentration x in ng per injection).

Some chromatograms of serum samples are presented in Fig. 7.

Detection limit. As determined by elution of a 1 ng/ml sample (Fig. 7a), the

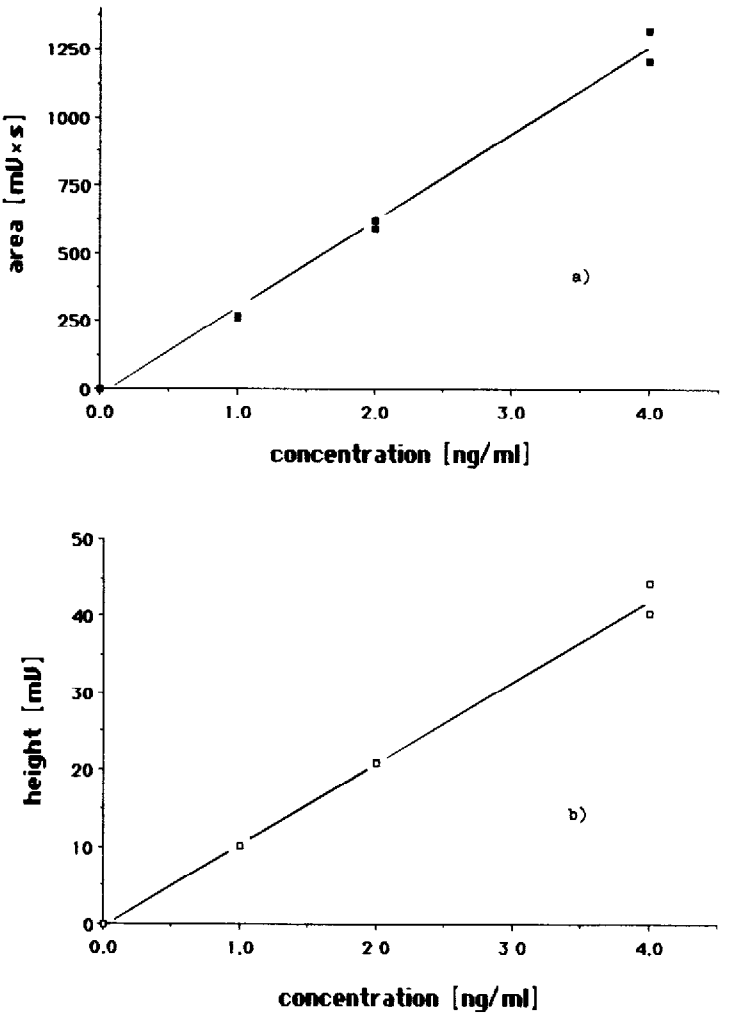

Fig. 6. Calibration plots for digoxin quantification in sera: (a) peak area versus concentration; (b) peak height versus concentration.

detection limit is 300 pg/ml (injection volume, 400 μl). This limit is reasonably good and allows determinations over the relevant concentration range of 0.5–2.0 ng/ml. The higher detection limit compared with that observed in analysis of pure analytes may be due to the longer elution time or to fading of detector sensitivity.

Reproducibility. The reproducibility is not as good as in elution of pure analytes. The reason may be the increase in the detection limit. The R.S.D. for the peak area is 6.9% ($n=12$, 2 ng digoxin per ml) and 6.5% for the peak height ($n=12$, 2 ng digoxin per ml). Since it has been demonstrated that recovery from the serum sample is quite high, the decrease in precision appears to be primarily a result of the reaction detector. Despite this decrease, the measurements permit a confidence range of nearly 10% (for duplicate analyses), which is reasonably good for this type of quantification.

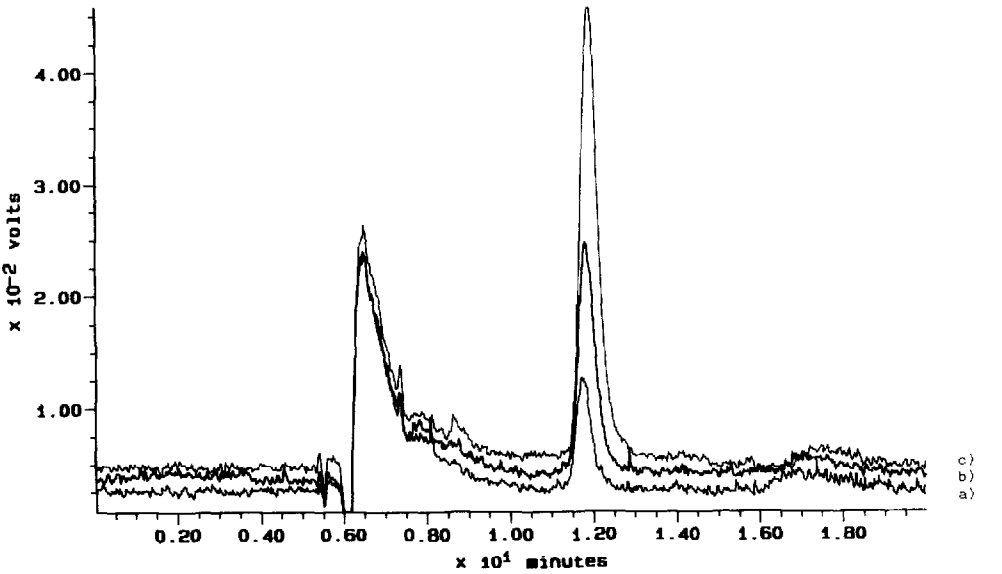

Fig. 7. Elution of serum samples: (a) 1.0 ng digoxin per ml; (b) 2.0 ng digoxin per ml; (c) 4.0 ng digoxin per ml.

TABLE II

RECOVERY RATES FOR DIGOXIN IN VARIOUS SERUM SAMPLES

Digoxin concentration, 2 ng/ml.

Serum sample	Recovery rate (%)	Remarks
Pool serum	73.7	Rather turbid, heterogeneous
Precinorm U	82.0	Normal
Precinorm L	80.7	Normal lipid content
Precilip	96.4	Normal
Precilip E.L.	82.9	High lipid content
Precipath U	74 2	Turbid

Recovery. Determination of the recovery rate is one way to evaluate the accuracy of a method; another is to compare it with a reference method. Although such an independent procedure is not currently available in this case, valuable indications may be obtained by spiking various serum samples and comparing the recovery rates. As shown in Table II, the recovery rate is not identical in all cases. It is particularly depressed in the case of samples that are turbid and contain coagulated serum proteins. None of the samples was filtered prior to injection, but it is advisable to do so to avoid rapid clogging of the injection column when a long series of unattended routine analyses are performed. This operation will inevitably lead to loss of analyte in such cases. It may be noted that this problem is not peculiar to the immunoadsorptive clean-up procedure.

Selectivity. The elution pattern in Fig. 7 shows that the chromatogram has only one extraneous peak at the start. Isocratic elution produces no foreign peaks

within the elution range. This permits determination of separated analogues and metabolic analytes. Several elutions of various blank samples are presented in Fig. 8 as a confirmation of this desirable feature.

Fig. 9a and b illustrate elution of a serum sample with and without purging. The extraneous peak is not significantly different in the two chromatograms, and the range of interest is as clean without purging as when purging is carried out with aqueous methanol. It is generally preferable to omit purging where possible to reduce the amount of equipment involved.

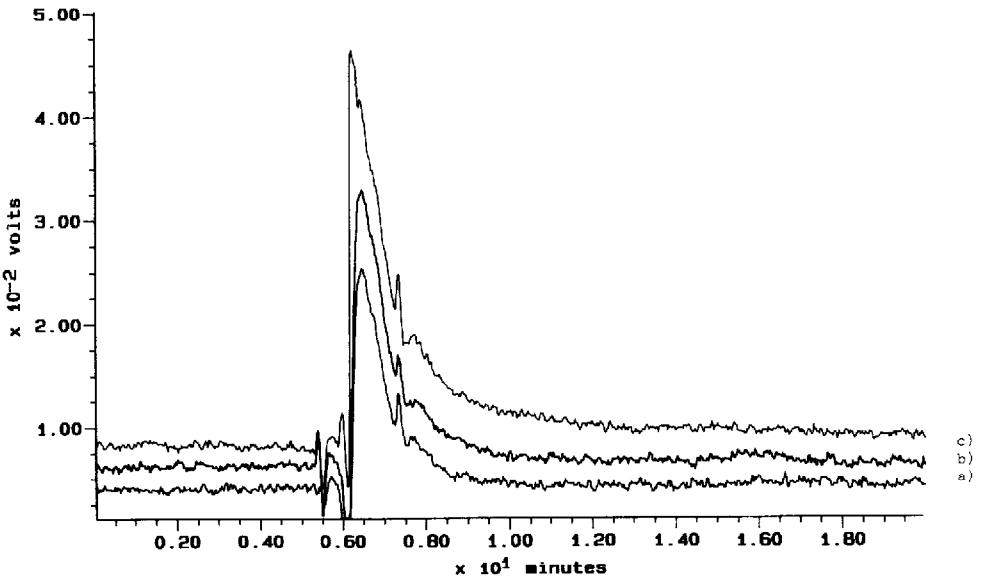

Fig. 8. Chromatograms of blank serum samples.

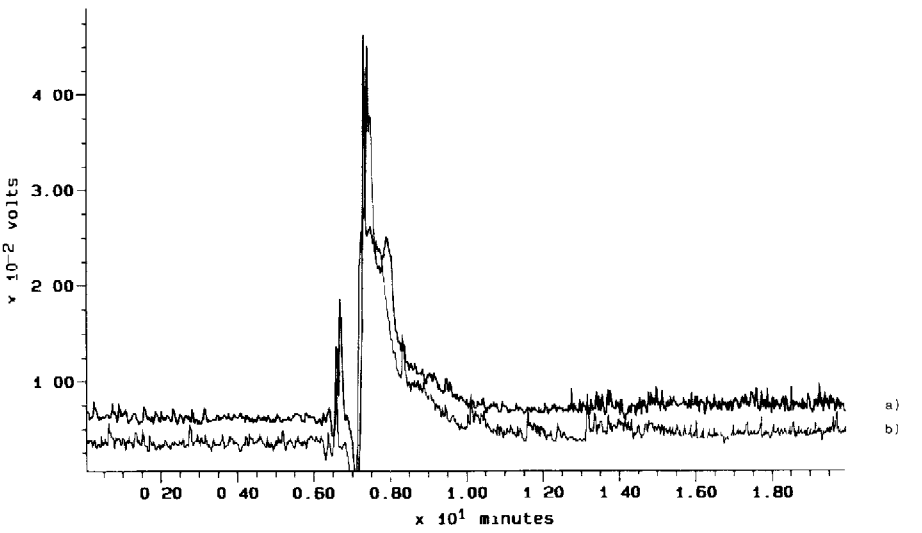

Fig 9. Chromatograms of serum samples (a) with and (b) without purging.

TABLE III

COMPARISON OF NEW, USED AND OLD IMMUNOADSORBERS

Adsorber	Peak area	Recovery rate (2 ng/ml of serum)
New (equilibrated with 1 ml of BSA solution)*	1212 mV s	100%
Used (ca. 6 ml of serum already injected)	1225 mV s	101%
Old (ca. 24 ml of serum already injected)	1095 mV s	90%

*Newly packed adsorbers must be equilibrated with 1 ml of BSA solution (50 mg of BSA per ml of 10 m*M* phosphate buffer, pH 6.8) to prevent unwanted spikes in the initial chromatograms.

Stability. Repeated injections of pure analytes confirm their long-term stability under the analytical conditions. Injection of large volumes of serum (400 μl per injection) would be expected to stress the immunoadsorber. The recovery rates obtained with a new, a used and an old clean-up cartridge are compared in Table III. It is apparent that repeated sorption and desorption of serum samples does not interfere with the recovery rate up to injection volumes of 10–15 ml. It is thus advisable to change the injection cartridge daily, particularly since this also avoids increased back-pressure due to clogging.

CONCLUSIONS

The data presented here demonstrate the possibility of selective and simple clean-up in complex matrices by use of on-line immunoadsorption prior to chromatographic elution. The performance of this technique, in terms of the recovery rate, selectivity and stability, is quite acceptable for routine use. Despite the use of a special sorbent, the commercial availability of defined antibodies for most of the relevant biological analytes makes this clean-up technique widely applicable. Using this clean-up technique, it may also be possible to analyse other substances (such as triiodothyronine), which currently cannot be determined with conventional HPLC methods.

Further work is required to assess the possibility of determining various analytes (such as digoxin/digitoxin or antiepileptic drugs) in a single run by combining several immunoadsorbers for clean-up and separation of the species in gradient elution.

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