# Determination of Digoxin in Human Serum by LC/MS with Online Sample Preparation

## Application Note

H. Sudergat, K. K. Unger, J. Emmert, J. Wendt, F. Mandel

### Introduction

Digitalis glycosides are the classic drugs for the treatment of congestive heart failure. Of these, digoxin (Figure 1) is one of the most prescribed cardiac glycosides. Although digoxin has been used for more than 200 years for the therapy of heart failure, it is still not possible to prepare digoxin synthetically. Digoxin is extracted from the leaves and seeds of the *Digitalis lanata* (foxglove).

Digoxin has a narrow therapeutic range of 0.5 to 2.0 ng/ml. Determination of digoxin in human serum is commonly performed by immunoassays (RIA, FPIA, ELISA) based on their sensitivity, speed, and low costs; but these assays lack specificity. Quantification can also be performed by HPLC with UV or fluorescence detection. Each of these techniques, however, has drawbacks. Due to the low extinction coefficient, UV detection without derivatization is not sensitive enough. For fluorescence

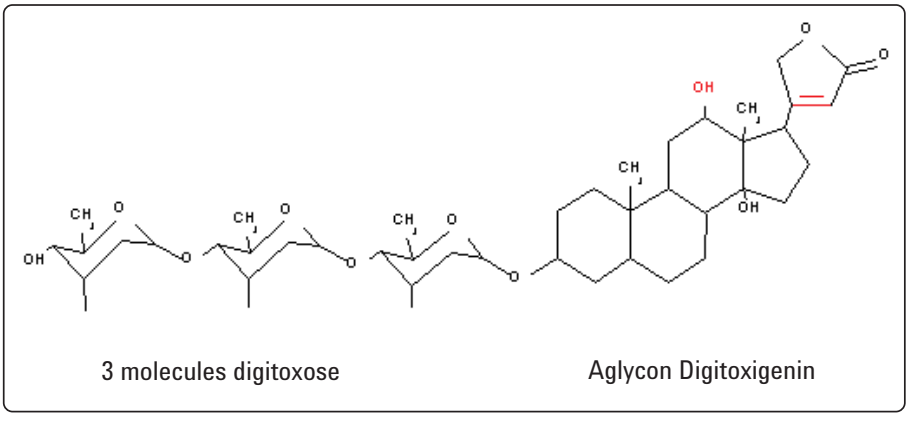


**Figure 1. Chemical structure of digoxin**

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detection, pre- or post-column derivatization is also required. In most of these cases, sample preparation is carried out off line with solid-phase extraction.

This application note describes a method for quantification of digoxin using liquid chromatography/mass spectrometry (LC/MS) with integrated online sample preparation that avoids the derivatization required by other HPLC methods.

## Experimental

The system was comprised of an Agilent 1100 Series binary pump, vacuum degasser, autosampler, thermostatted column compartment with a column switching valve, diode-array detector, and LC/MSD mass spectrometer. The LC/MSD was equipped with electrospray (ESI) and atmospheric pressure chemical ionization (APCI) ion sources. Complete system control and data evaluation was done on the Agilent ChemStation for LC/MS.

The integrated sample preparation was done on a precolumn packed with LiChrospher ADS RP4, a restricted-access material (RAM). This material (see Figure 2) combines a hydrophilic external surface (diol groups) and a hydrophobic internal surface (butyl groups) with a defined pore size. Matrix proteins from the sample are not able to enter the pores (size exclusion) and are not well retained by the external surface of the column material.

With the column switching valve in position 1, the matrix proteins eluted to waste (Figure 3) while the analyte accumulated on the precolumn. After the matrix proteins were washed away, the valve and mobile phase were switched. Backflushing the precolumn with mobile phase B (higher content of organic solvent) transferred the analyte to the analytical column. Because the resulting transfer volume was much smaller than the injection volume, this had the effect of concentrating the analyte.

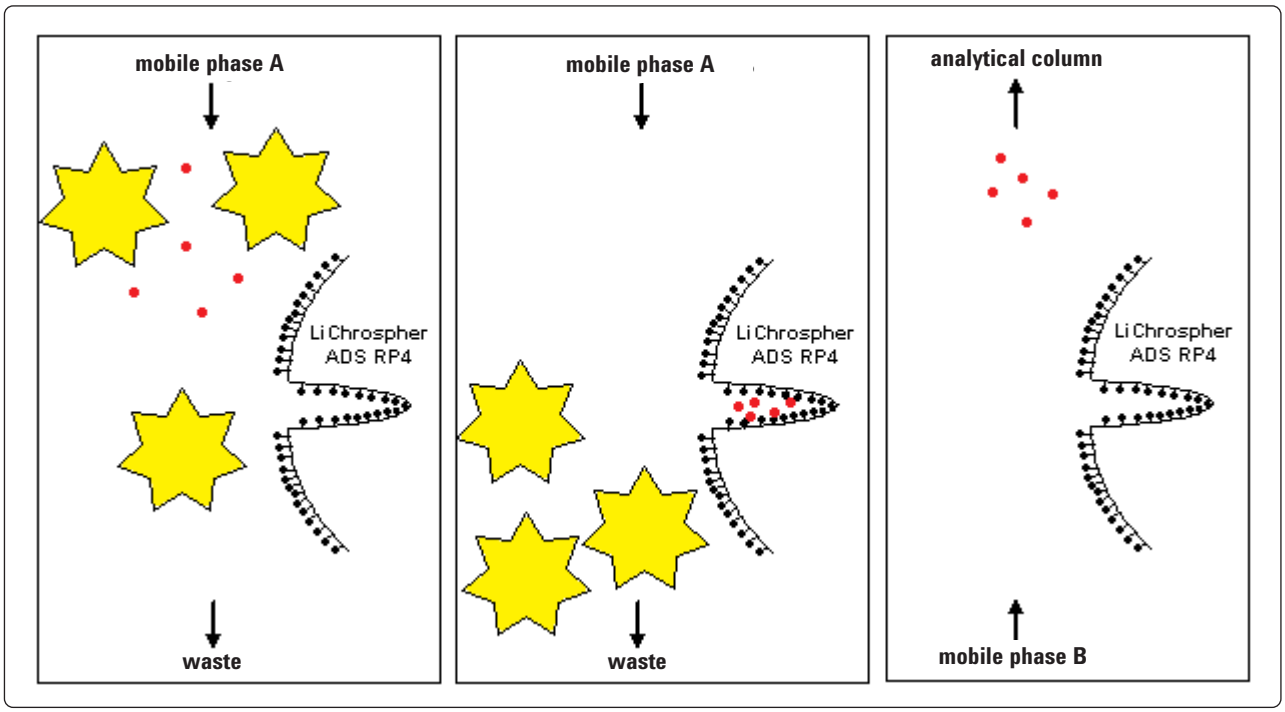



**Figure 2. Principle of LiChrospher ADS RP4 in the sample preparation of digoxin**

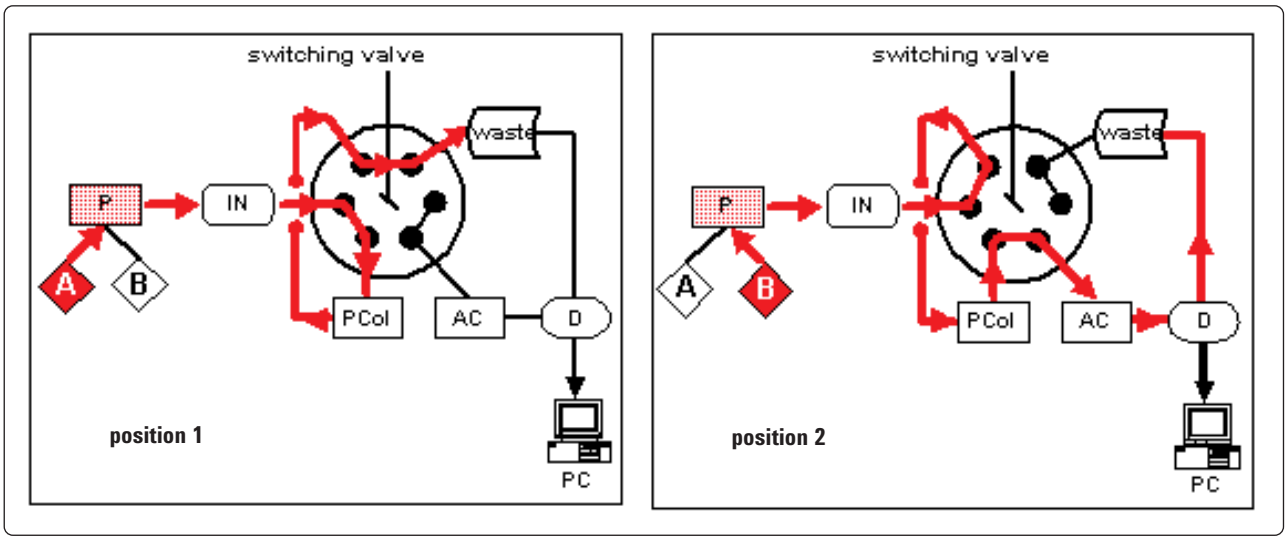



**Figure 3. Switching valve positions: position 1 = sample preparation; position 2 = analyte transfer to analytical column by backflushing. A = mobile phase A, B = mobile phase B, P = pump, IN = injector, PCol = precolumn, AC = analytical column, D = detector, PC = personal computer (data system)**

The separation of digoxin and its metabolites was done by isocratic elution on a Hypersil SAS column (5 µm, 125 mm x 3 mm) with a mixture of sodium citrate (1 mM):methanol:ethanol: isopropanol (50:46:3:1 (v/v)). Due to their small molar absorption at UV wavelengths less than 220 nm, direct detection of the digoxin and its metabolites without derivatization was only possible with MS detection.

## Results and Discussion

Both APCI and electrospray were used to analyze digoxin. Using APCI, the intensity of the protonated molecule (*m/z* 780) was low and a high degree of fragmentation was observed (see Figure 4). Successive losses of digitoxose molecules (Δ = *m/z* 130) resulted in a series of characteristic, evenly spaced mass peaks.

Electrospray ionization with 1 mM ammonium acetate in the mobile phase yielded neither the protonated molecule nor the ammonium adduct

**ANALYSIS METHOD**

**Conditions**

| | |
|---|---|
| Precolumn: | LiChrospher ADS RP4, 25 µm, 25 x 4 mm (E. Merck, Darmstadt, Germany) |
| Analytical column: | Hypersil SAS, 5 µm, 125 x 3 mm (MZ-Analysentechnik, Mainz, Germany) |
| Mobile phase: | A = Methanol/Water (3/97)<br>B = 1 mM sodium citrate/ methanol/ethanol/isopropanol (50/46/3/1) |
| Gradient: | 0–20 min at 100% A<br>20–50 min at 100% B |
| Post time: | 5 min (reconditioning precolumn with 100% A in switching valve position 1) |
| Flow-rate eluent: | 0.5 ml/min |
| Column-switching settings: | 0–20 min: Position 1 (Figure 2)<br>20–50 min: Position 2 (Figure 2) |
| Post time: | 5 min |
| Temperature: | 35°C |
| Injection volume: | 100 µl |
| Detection: | MS |
| Interface: | ESI or APCI |
| Polarity: | Positive |
| Drying gas temperature: | 325°C |
| Drying gas flow: | 10 l/min |
| Nebulizer pressure: | 50 psig |
| Fragmentor: | 180 V |

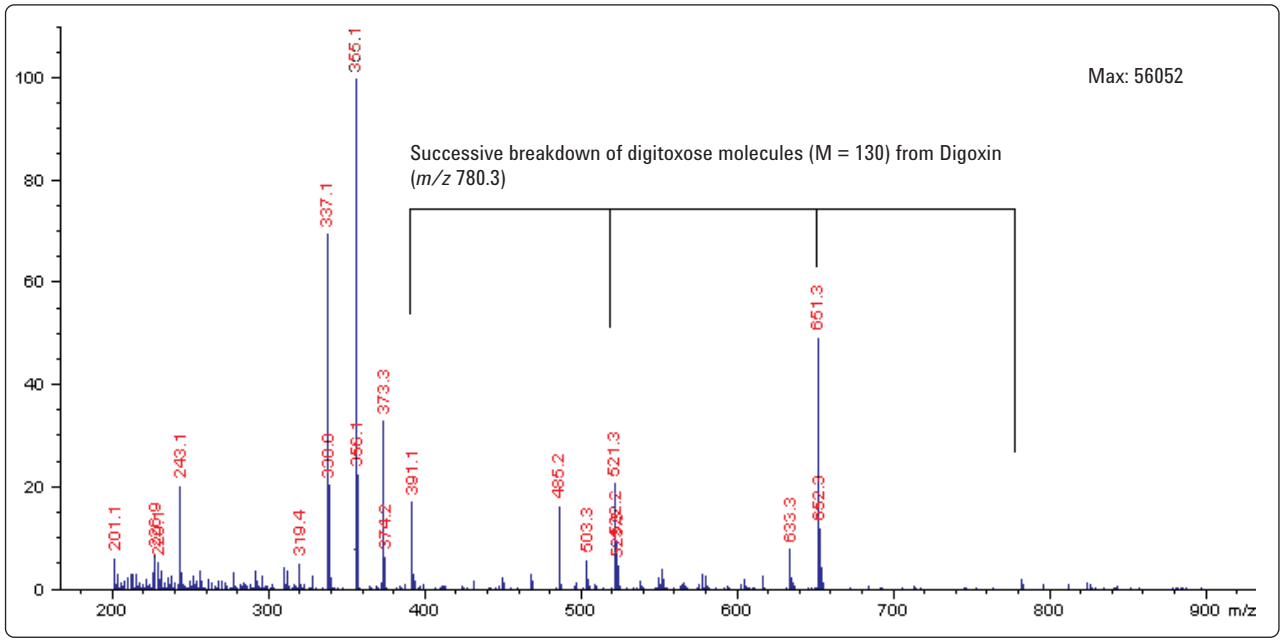



**Figure 4. Mass spectra of digoxin acquired using atmospheric pressure chemical ionization**

but instead showed a sodium adduct [digoxin+Na]$^+$ at *m/z* 803.5 (see Figure 5). This was likely due to trace levels of sodium present from leaching of glass vials or bottles or from impurities in the mobile phase. Substituting 1 mM sodium citrate for the ammonium acetate enhanced the formation of the stable sodium adduct and did not significantly affect the chromatography. Using the stable sodium adduct, selected ion monitoring was used to detect digoxin with a very high degree of sensitivity.

A limit of quantification (LOQ) of 0.5 ng digoxin per ml of human serum (c.v. 2.8%) was determined by injecting 100 µl of spiked human serum without any sample preparation except centrifugation (Figure 6). This corresponds to the detection of 0.05 ng digoxin absolute on column.

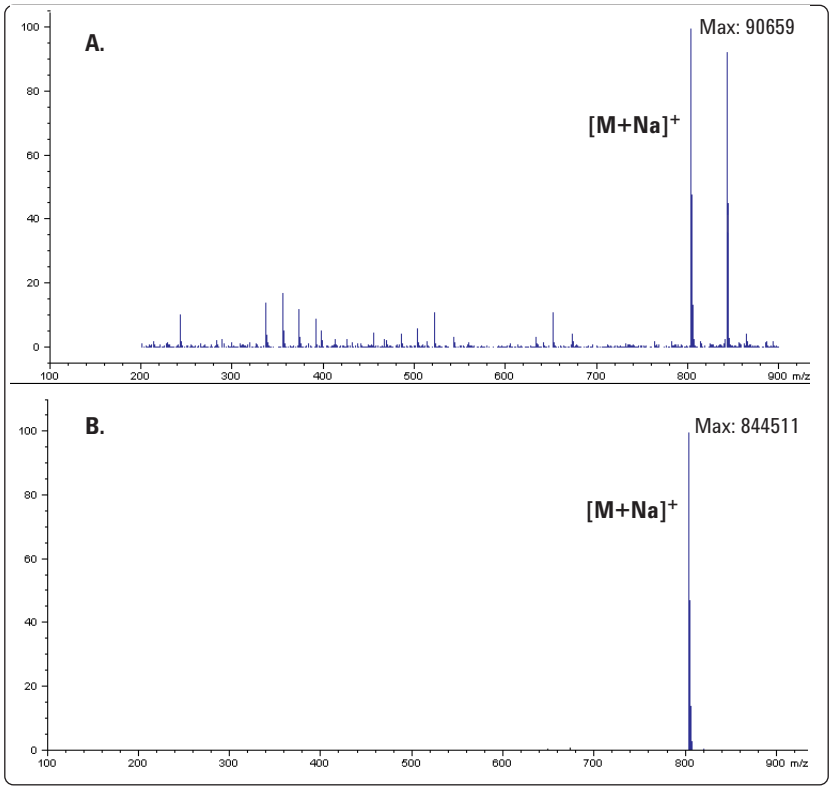
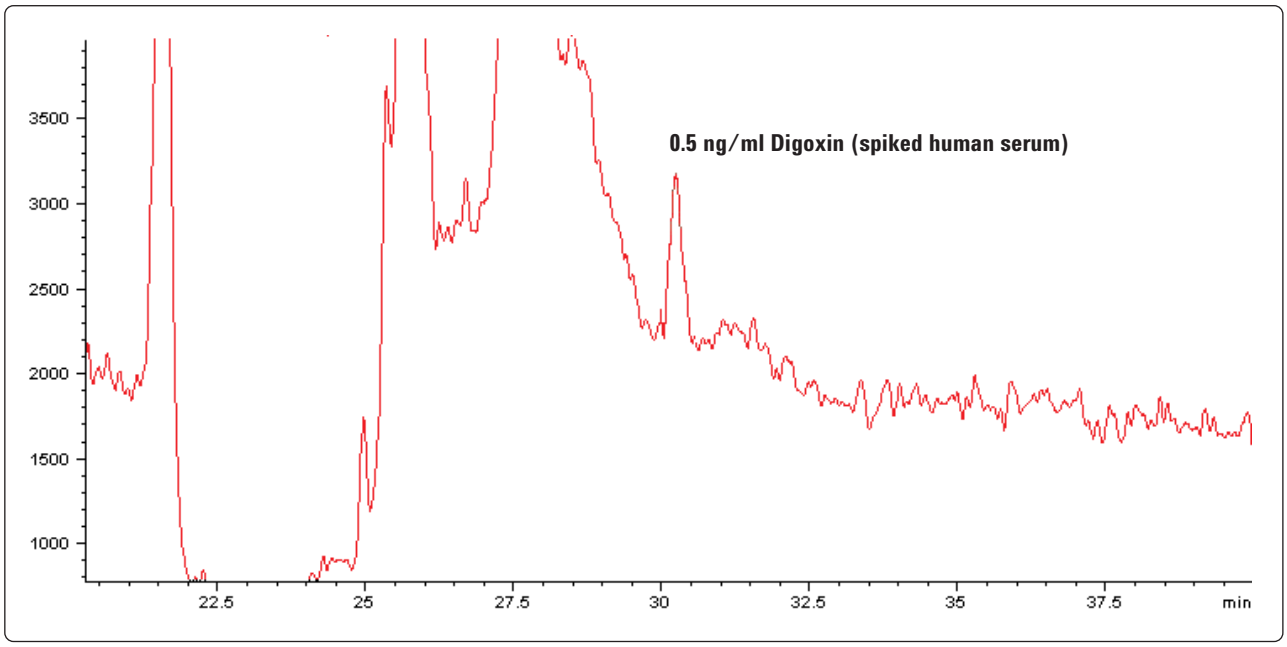



**Figure 5. Mass spectra from electrospray ionization of digoxin using: (A) 1 mM ammonium acetate buffer and (B) 1 mM sodium citrate buffer**

**Figure 6. Chromatogram of digoxin in human serum at the limit of quantification**



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