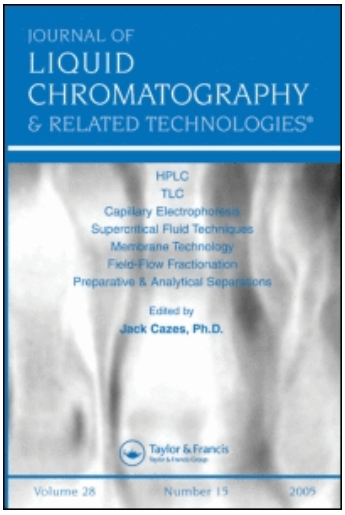


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# TENSAMMETRIC DETECTION IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. APPLICATION TO LYNESTRENOL AND SOME CARDIAC GLYCOSIDES

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## ABSTRACT

The application potential of tensammetric flow-through detection in high-performance liquid chromatography is studied. Batch experiments are performed to obtain optimal detection potentials. Lynestrenol, a steroid hormone used for birth control and the cardiac glycoside digoxin are used as model compounds. Detection limits have been found in the order of 20 ng per injection and permit the analysis of low dosage of pharmaceutical formulations. The operation of the flow-through tensammetric detection system is tested by detecting six cardiac-glycosides after reversed-phase chromatographic separation. For these analytes direct tensammetric detection has been shown to be a feasible technique. The use of such adsorption properties at a mercury electrode has potential as a complementary electrochemical detection technique for certain groups of compounds with no conventional electrochemical activity.

## INTRODUCTION

The application of electrochemical flow-through detectors in HPLC based on the dropping mercury electrode (DME) has been demon-

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strated by several workers [1-4]. These DME detectors were mostly used in the reductive mode. Some studies reported [5,6] on the application of tensammetry as detection technique in chromatographic systems. Tensammetry allows the detection of electro-inactive compounds that adsorb at the mercury electrode surface. In batch experiments a wide range of compounds have been studied [7-9]. Several theoretical aspects of tensammetry in non-flowing systems were subject to extensive treatment by various authors [7-12].

A classical tensammogram [7,8] is presented in Fig. 1, it shows two adsorption-desorption waves and baseline depression with respect to the blank electrolyte between these waves. Not only the heights but also the position of the adsorption-desorption waves in the tensammogram depends on the analyte concentration [7]. This makes the use of the adsorption-desorption process troublesome for detection in continuous flow systems when using commercially available AC polarographic equipment. By introducing computer operated devices or scan techniques as used in batch experiments on both capacitive and faradaic processes, this problem can be overcome [13,14]. However, the most straight foreward approach to the application of tensammetry to continuous flow systems is the use of the properties in the adsorbed state. Adsorption of the analyte causes a depression in the electrochemical double layer capacity. This change can be measured by monitoring the double layer capacity on a display device [5,15] or by monitoring the capacity current [6]. When using the latter method calibration of the measuring device is not necessary. The double layer capacity depression can be observed over a wide potential range. The strongest depression is found in the neighbourhood of the electro-capillary maximum. This position is not specific for the analyte studied. Because of this property of the adsorption process, Kemula claimed tensammetry to be the most universal electrochemical detection technique [5].

Besides the usual limitations in eluent choice introduced by the demands of electrochemical detection, one is, when operating a ten-

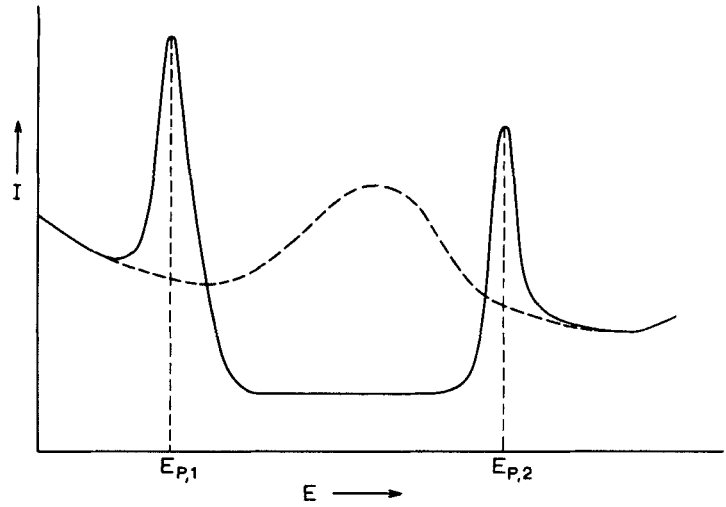



Fig. 1. Schematic representation of a tensammogram. $E_{p,1}$: peak potential of the positive tensammetric wave. $E_{p,2}$: peak potential of the negative tensammetric wave. ——— tensammetric curve ----- blank curve



sammetric detector in reversed-phase high-performance liquid chromatography (RP-HPLC), faced with adsorption of the polarity modifier at the electrode surface. Methanol and acetonitrile seem to be the most suitable modifiers in this context. Ethanol and higher alcohols adsorb so strongly that only a few percent in a RP-eluent can be tolerated. In general, addition of a polarity modifier gives rise to a loss in signal. A compromise is therefore often necessary between optimal separation and optimal detection conditions.

In this study the application of a tensammetry based detector is illustrated by the detection and separation of several cardiac glycosides and by the detection of lynestrenol (19-Nor-17α-pregn-4-en-20-yn-17-ol), a steroid hormone used in formulations for birth regulation. In literature the chromatographic behaviour of cardiac glycosides was studied in both normal-phase and reversed-phase systems

[16-19]. Most determinations of lynestrenol were carried out with TLC and GC methods [20-23]. No HPLC data are available on lynestrenol because of detection problems. Using a tensammetric detector we shall demonstrate in this paper that RP-HPLC can be operated for the determination of the compounds mentioned in pharmaceutical formulations.

## EXPERIMENTAL

For obtaining the most suitable detection potential complete tensammograms were recorded in batch with a classical polarographic setup. In continuous flow and chromatographic systems a PAR 310 electrode or a polarographic HPLC detector developed by Hanekamp et al. [24] was used. Both detectors enable synchronization of drop--time and electronics, hence sampled AC measurements can be performed.



### Apparatus

A Princeton Applied Research (PAR) model 174 polarograph and a PAR 129A lock-in amplifier (EG & G, Princeton Applied Research Co., Princeton, NY, USA) both modified by our workshop for sampled AC operation were used with the PAR 310 static mercury drop electrode. These devices were interconnected with a PAR 174/50 AC polarographic interface. A Peekel 053A sinewave oscillator was used for generating the alternating voltage. The frequency of the alternating voltage was measured with a HP 5300A measuring system (Hewlett-Packard, Colorado, USA). A Fluke 8000A digital multimeter (John Fluke, MFG Co. Inc., Washington, USA) was used for checking the dc-detection potential. The overall current was monitored on a Tektronix 5103A oscilloscope (Tektronix, Beverton, OR, USA). The current was recorded on Kipp BD8 multirange recorder (Kipp en Zonen, Delft, The Netherlands).

In the second setup, the home-made detector [24] was connected with a Bruker E 310 modular research polarograph (Bruker Spectrospin

S.A., Brussels, Belgium). A PAR model 175 programmer was used for generating a drop dislodge pulse as described elsewhere [24]. The tensammograms were recorded on a HP 7046A XY-recorder (Hewlett-Packard). The chromatograms were recorded on a Servo 901 RE 571 Y-t recorder (Goerz Electro GmbH, Vienna, Austria). The overall current was monitored on a Tektronix type 502 oscilloscope. The applied potentials were always measured vs. a Ag/AgCl/1 M LiCl methanol-water 50/50% v/v reference electrode. In both chromatographic systems a PE 601 pump (Perkin-Elmer, Connecticut, USA), a Rheodyne 7120 injection valve (Rheodyne, Inc., Berkeley, CA, USA) and a stainless-steel column (10 cm x 4.6 mm I.D.) were used. The columns were packed with ODS Hypersil 5 µm (Shandon, Rumcorn, UK) or LiChrosorb RP-2 10 µm (Merck, Darmstadt, GFR).



## Chemicals

The measurements were performed in water-methanol mixtures containing 0.1 M $KNO_3$. The stock solutions were deaerated by purging with nitrogen (A28). Water was dimineralized and distilled. The cardiac glycosides were supplied by Sandoz Ltd, Basel, Switzerland and Lynestrenol by Organon Ltd., The Netherlands. All other chemicals were analytical reagent grade (Baker "Analyzed" or Merck p.a.). The samples were deaerated by purging with nitrogen for about 10 minutes.

# RESULTS AND DISCUSSION

## Batch Experiments

Complete tensammograms of digoxin and lynestrenol were recorded as a function of the methanol-water ratio in the supporting electrolyte. In Fig. 2 some results obtained for digoxin are presented. The current depression (analytical signal) by adsorption of digoxin measured at a constant potential (-400 mV vs. Ag/AgCl) was observed to decrease considerably in going from 0% methanol ($\Delta I$ = 3.83 µA) to 80% ($\Delta I$ = 0.54 µA). This potential is chosen from

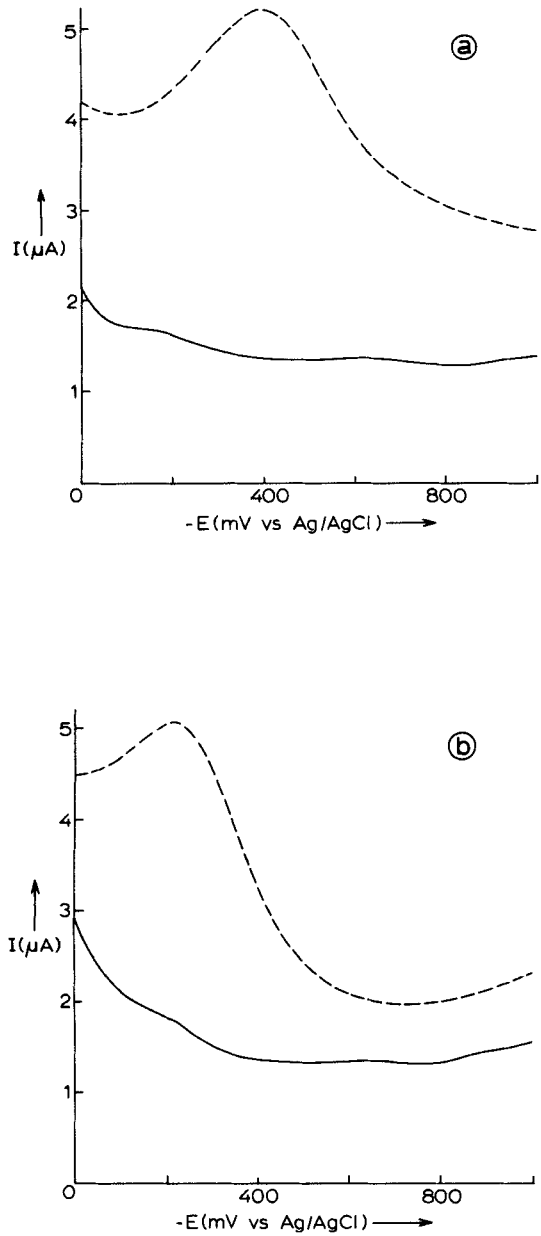
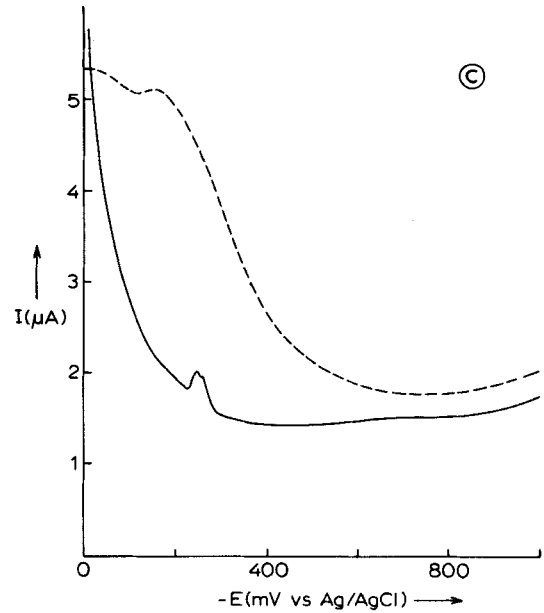
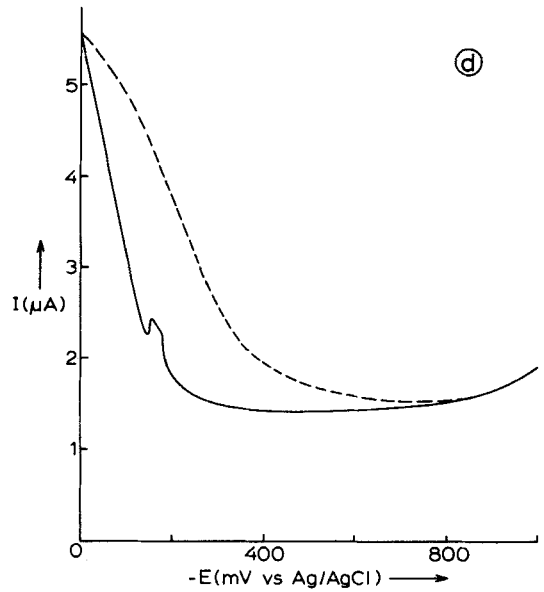



Fig. 2. Tensammograms of different electrolyte composition without (----) and with (——) $10^{-4}$ M digoxin. Conditions: f = 60 Hz, amplitude 20 $mV_{eff}$, $\phi$ = 90 degrees, $t_d$ = 1.0 s, $h_{Hg}$ = 50 cm, $\phi$ capillary = 0.010 cm (length 10 cm), scan




rate 2 mV $s^{-1}$. Electrolyte compositions: 0.1 M $KNO_3$ in various (v/v) ratios water-methanol:

a. 100% water

b. 70%-30% water-methanol

c. 50%-50% water-methanol

d. 20%-80% water-methanol

the tensammogram displayed in Fig. 2a. At this potential the current reaches a maximum value which is caused by a change of the double layer capacity due to specific adsorption and desorption of the anions from the supporting electrolyte [9].

Methanol addition to the electrolyte induces a change in position of this maximum in the tensammogram as can be seen from Fig. 2a and 2b. When the relative amount of methanol is increased even further the characteristic maximum disappears completely as can be seen from Fig. 2c and 2d. From Fig. 2 it can be concluded that various electrolyte compositions have different optimal detection potentials. Even at optimal detection potentials a net decrease in current depression and hence in sensitivity is observed when changing from a purely aqueous electrolyte towards larger amounts of methanol in the electrolyte. At methanol concentrations exceeding 60 percent (v/v) this effect is less obvious; then methanol adsorption is almost completely governing the capacity-potential curve. Lynestrenol qualitatively shows the same behaviour. The current depression is less strong as for digoxin in the potential range considered. The optimal detection potentials for lynestrenol in the electrolyte compositions studied differ approximately 50 millivolts from those for digoxin.



## Continuous Flow Experiments

In a continuous flow system the peak height, which is actually the depression of the baseline was found to be linearly dependent on the applied modulation-frequency and the modulation amplitude. In Fig. 3 the peakheight (Ip) versus phase-angle ($\phi$) is plotted for digoxin. From this graph it can be seen that the maximum current depression is observed at about 30 degrees with respect to the applied modulation voltage. This is a large deviation from the theoretical expected 90 degrees. In similar experiments with lynestrenol, performed with the PAR 310 detector, the maximum baseline depression was found between 80-100 degrees, which is more acceptable [9]. These observations suggests that the cell geometry might be responsible for the phase angle difference observed.

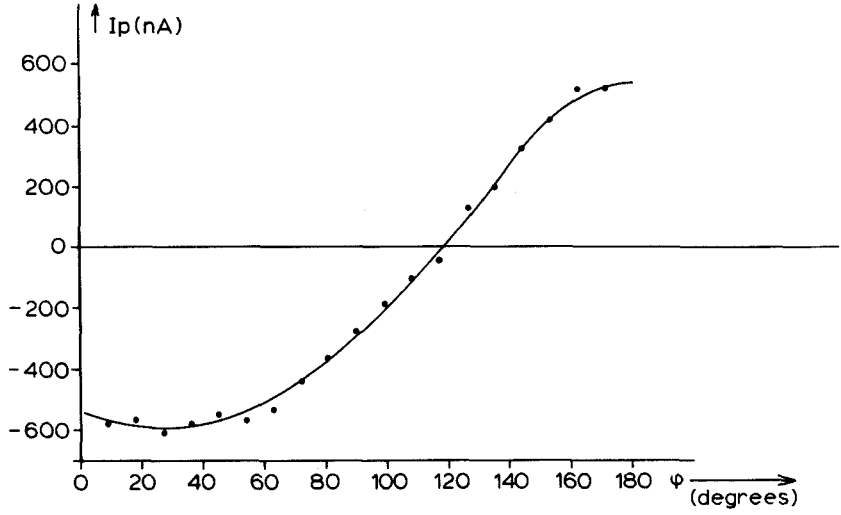



Fig. 3. Peak height ($I_p$) vs. phase-angle ($\phi$) in continuous flow system with the home made DME detector for 200 µl injections of $10^{-4}$ M digoxin. Conditions: f = 35 Hz, amplitude = 30 $mV_{eff}$, detection potential = -150 mV vs. Ag/AgCl, $t_d$ = 1 s, $h_{Hg}$ = 30 cm. Eluents: water-methanol 40%-60% (v/v) containing 0.1 M $KNO_3$. Flow rate: 1 ml/min.



## Application to HPLC

The application of continuous flow tensammetric detection was studied with two HPLC systems. Detection limits, linear dynamic range and precision were investigated with the PAR apparatus. Six cardiac glycosides were detected, after separation with the Bruker polarograph.

Results for lynestrenol: Lynestrenol was chromatographed in a RP--system using a LiChrosorb RP2 10 µm packed column and a flow rate of 2 ml/min (30%-70% (v/v) water-methanol, 0.1 M $KNO_3$). The capacity factor k' was 3.2. The detection potential was chosen from batch experiments to be -150 mV vs. Ag/AgCl. From Fig. 2a it can

be seen that dI/dE is large at this potential in a blank electrolyte solution. Availability of a more stable potentiostat might have been resulted in a smaller baseline noise caused by potentiostat instability. Fig. 4 shows a logarithmic plot of the calibration curve for lynestrenol. In the range from $2 \times 10^{-6}$ M, $2 \times 10^{-4}$ M, a linear relationship exists between peak height and concentration. In this region a calibration line is calculated using the method of least-squares. The regression coefficient was 0.996 and the sensitivity was $8 \times 10^{-4}$ AL/mole. At concentrations exceeding $2 \times 10^{-4}$ M deviation from linearity is observed. In this region saturation of the electrode surface with lynestrenol starts to become important. In our setup a typical noise of 1 nA was observed. The detection limit for lynestrenol (M = 284.42) at a signal to noise ratio of 3:1 was calculated to be $1.0 \times 10^{-6}$ M or 28 ng per injection. The standard deviation of peak height for 11 consecutive injections (1.7 µg lynestrenol per injection) was 2.4%.



Results for Cardiac Glycosides: Cardiac glycosides were chromatographed in a RP system using an ODS Hypersil 5 µm packed column at a flow rate of 1 ml/min (40%-60% (v/v), water-methanol, 0.1 M $KNO_3$). The detection limit (conditions see Fig. 5) for digoxin (M = 780.92) was found to be $1.0 \times 10^{-7}$ M or 16 ng per injection (with typical peak height 3 nA). The capacity factor for digoxin was 3.5. In Fig. 5 a HPLC separation of six cardiac glycosides is presented. Note the peak height differences between digitoxin and gitoxin on one hand and the other compounds on the other hand. These differences are caused by differences in adsorption strength at the detection potential chosen.

Application to Pharmaceutical Formulations: The potential of this detection technique for the analysis of pharmaceutical formulations was tested with digoxin ampoules and tablets. The chromatographic and detection conditions were the same as for Fig. 5. Ampoule solutions 20 µl, were injected directly without any further pretreatment. The tablets containing 0.25 mg digoxine were pulverized with a mortar. After shaking for a few minutes with a mix-

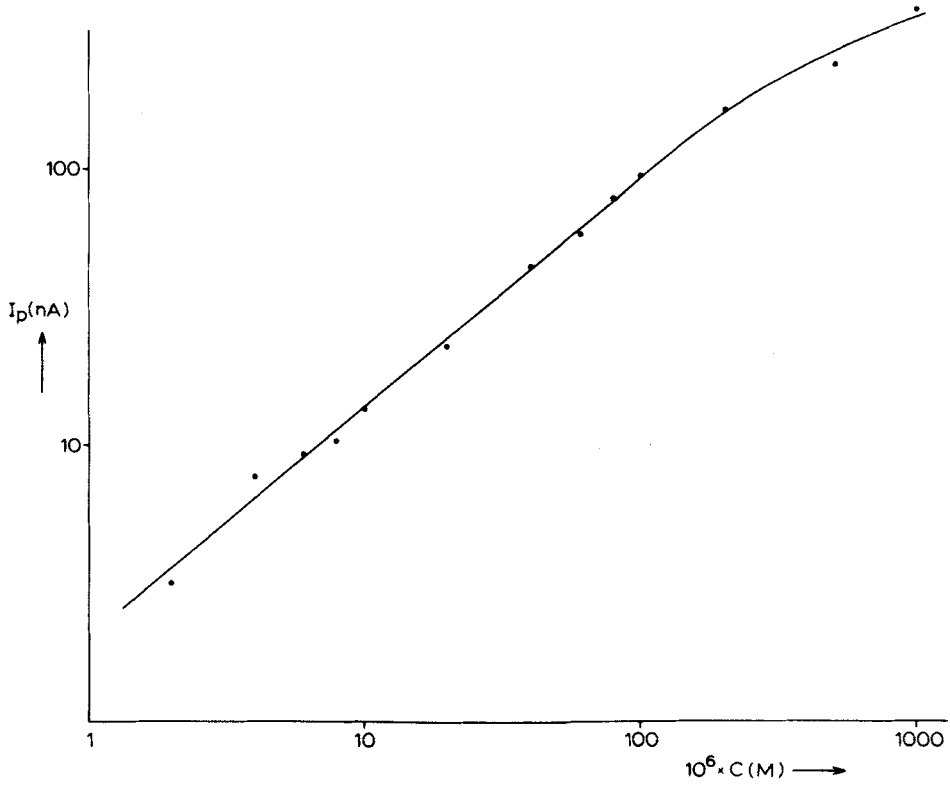



Fig. 4. Peak height ($I_p$) vs. concentration lynestrenol. Conditions: f = 20 Hz, amplitude 10 $mV_{eff}$, $\phi$ = 85 degrees, detection potential -200 mV vs. Ag/AgCl, $t_d$ = 2 s, drop size: large (r = 0.0935 cm), time constant = 3 s. Injection volume: 100 µl. Eluent: water-methanol 30%-70% (v/v) containing 0.1 M $KNO_3$. Flow rate = 2 ml/min.

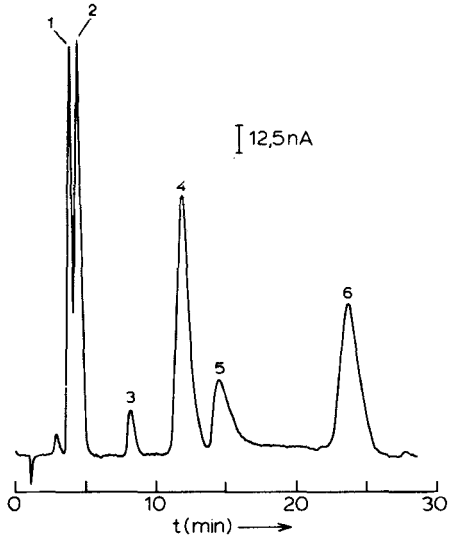



Fig. 5. Chromatogram of 6 cardiac glycosides: (1) digoxin, (2) digitoxin, (3) gitoxin, (4) lanatoside A, (5) lanatoside B, (6) lanatoside C. 20 µl of a mixture containing $10^{-4}$ M of each compound was injected. Detection conditions: $h_{Hg}$ = 40 cm, time constant 2.5 s. Further conditions see Fig. 3.

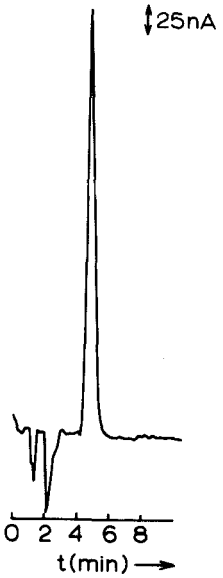



Fig. 6. Chromatogram of 0.25 mg digoxin tablet. Conditions: $h_{Hg}$ = 18 cm, $t_d$ = 0.8 s, further conditions see figure 5.

ture of EtOH 0.25 ml, MeOH 0.25 ml and $H_2O$ 0.5 ml, 20 µl of the supernatant liquid were injected. A chromatogram for a digoxin tablet is shown in Fig. 6. Detection limits and reproducibility are comparable to those reported for standard solutions.

## CONCLUSIONS

Tensammetric detection in HPLC is a feasible complementary technique to oxidative and reductive electrochemical detection. It is particularly favourable for groups of compounds which do not possess a strong chromophore. Lynestrenol is a good example in this regard. For cardiac glycosides the detection limits obtained are about comparable to UV detection at 220 nm [25] and thus no advantage would be gained by using this technique for formulations analysis, although some improvements could be made as to using more stable potentiostats. From the experience gained in this work we tend to agree with Kemula and Kutner [5] that tensammetric detection can be more universally applicable than other electrochemical detection modes. Further work is needed to explore other potentially interesting groups of compounds.



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