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ALTERNATING VOLTAGE POLAROGRAPHIC DETECTION FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS EVALUATION FOR THE ANALYSIS OF BILE ACIDS

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SUMMARY

The first attempt to apply alternating voltage (a.v.) polarography for detection in high-performance liquid chromatography is presented. The performance of a flow-through polarographic detector in the a.v. polarographic mode is illustrated with the separation of a mixture of bile acids. Generally, the retention time of a substance is found to be proportional to its extent of adsorption at the electrode at the applied potential. Thus, the more the substance is retained, the easier is the detection of its peak. The observed noise level is close to 0.024 μF^{-1}. The linear dynamic range for cholic acid determination extends from 1.25 nmol to over 50 nmol with a correlation coefficient of 0.998. The sensitivity is close to $8 \cdot 10^4$ sec l μF^{-1} mol^{-1} and the detector response index is 0.98. The standard deviation of the peak area from nine consecutive injections was 4.5%. The detection limit was close to 1 nmol per injection, almost one order of magnitude larger than that for a UV detector operated at 210 nm, but still one order of magnitude lower than that for a refractive index detector.

INTRODUCTION

The first attempt at applying alternating voltage (a.v.) polarographic detection[1,2] in high-performance liquid chromatography (HPLC) is presented in this paper.

Many organic and inorganic substances present in electrolyte solutions result in a change in the double layer capacity at the dropping mercury electrode (DME) due to their specific adsorption. This effect was extensively exploited for the determination of many organic compounds using alternating current (a.c.) polarography and the adsorption data for many systems are available[3]. The effect was also applied for detection in liquid chromatography, but until now only a.c. polarography has been used, in both total current[4–6] and phase sensitive[7] modes of operation. We found that a.v. polarography is also useful for monitoring HPLC signals.

EXPERIMENTAL

A Radelkis OH-105 universal polarograph (Hungary), operating at $\omega = 60$ Hz

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and amplitude of alternating voltage $\Delta E_{\sim} = 20$ mV, was used in a.c. polarographic measurements.

For a.v. polarographic experiments the home-made[2] PZN 71 a.v. polarograph was used which recorded the $1/C$ *vs.* E curves. This apparatus operated at 240 Hz in a "tast" mode in order to eliminate the oscillations due to the dropping mercury. The signal was sampled for 0.2 sec, after 1 sec of mercury drop age. A simple resistance–capacitance damping circuit having a time constant of 3 sec was also used.

The home-made HPLC apparatus with 5-μl injection valve[8] and the flow-through polarographic detector[8,9] used were described previously. The detector operated in the three-electrode mode with two 470-μF condensers connected together, with opposite signs between the auxiliary and reference electrodes. To verify the accuracy of experiments the resistance of the detector filled with the working solution was measured and compared with the highest acceptable resistance. For a capacity, C, of $\approx$0.2 μF the error in the measured $1/C$ values at $\omega = 240$ Hz was less than 10% for an electrolyte resistance, R, of $\leqslant$30 kΩ as measured using the equivalent circuit, somposed of capacitor and resistor in series; the resistance of the detector, when filled with a solution containing 60% methanol and 40% 1/15 M phosphate buffer, pH 6, according to Michaelis, was *ca.* 5.5 kΩ, for a drop time, t_1, of 1.6 sec, measured with the a.c. bridge[10].

The characteristics of the DME capillaries were as follows: (i) in steady-state a.v. and a.c. polarographic measurements (Fig. 1), $t_1 = 2$ sec, $m = 2.966$ mg sec^{-1}, $h_{Hg} = 117$ cm; (ii) in HPLC experiments (Fig. 2), $t_1 = 1.4$ sec, $m = 2.375$ mg sec^{-1}, $h_{Hg} = 40$ cm.

Analytical reagent grade chemicals and doubly distilled water were used. Bile acids were generously supplied by the Institute of Pharmaceutical Industry, Warsaw.

A 250 × 4 mm I.D. stainless-steel column was slurry packed at 435 kg m^{-2} with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.). The chromatographic procedure was as described previously[8].

RESULTS AND CONCLUSIONS

The steady-state experiments were performed with both a.c. and a.v. techniques.

The performance of the detector in a.v. polarographic mode may be illustrated with the separation of a bile acid mixture. The addition of bile acids to a methanol–water mixture resulted in an increase of the broad maximum in the $1/C$ *vs.* E a.v. polarographic curve (Fig. 1) due to specific adsorption. The potential of the maximum was practically independent of the nature and concentration of the added bile acid. This is why this potential is useful for analytical detection in HPLC.

As the detection limit of this method is dependent on the difference in double layer impedance, $1/C$, at the chosen potential in the absence and the presence of the studied substance, a suitable eluate for a.v. polarographic detection should reveal the lowest possible impedance value at this potential.

a.v. polarography appears to be advantageous for HPLC detection for several reasons. The equivalent circuit of the electrolytic cell in a.v. polarography comprises a capacitor and riesstance in series which corresponds to the scheme of connections

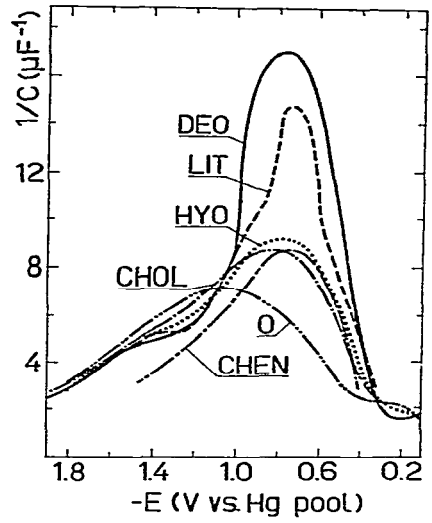

Fig. 1. Reciprocal capacity curves as recorded by a.v. polarography for 10^{-3} *M* bile acids in 0.1 *M* $NaClO_4$ solution containing 80% methanol and 20% 0.2 *M* acetate buffer, pH 4, according to Walpole. Names of steroids as in Fig. 2; O = no steroids.

in the electrochemical cell in the absence of faradaic reaction, contrary to a.c. polarography for which the equivalent circuit is a capacitor and resistance in parallel, which has no physical meaning. a.v. polarography is especially useful for measurements in electrolytes of low conductivity[2] and is the most sensitive for low capacities. The a.v. amplitude adjusts naturally to capacity variations, since the a.c. amplitude is kept constant. Finally, the calibration curve of $1/C$ a.v. polarographic output signal *vs.* concentration measured at the detection potential is more suitable for quantitative determinations than that of condenser current, i_C, output signal *vs.* concentration as recorded in a.c. polarography[11].

The HPLC separation of bile acids in 70% methanol–30% 0.2 *M* acetate buffer, pH 4 is presented in Fig. 2. Generally, the retention time of a substance was found to be proportional to the extent of its adsorption at the DME at the applied potential. Thus, the more retained the substance, the easier its peak detection. Until now, bile

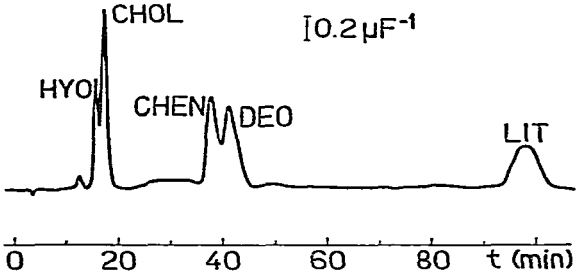

Fig. 2. HPLC chromatogram of 10^{-2} *M* bile acid mixture with a.v. polarographic detection. Separation conditions: stationary phase, 10-μm LiChrosorb RP-18; mobile phase, 0.1 *M* $NaClO_4$ solution containing 70% methanol and 30% 0.2 *M* acetate buffer, pH 4, deaerated with argon; mobile phase flow-rate, 1 ml min^{-1}; potential, $E = -0.5$ V *vs.* Ag/AgCl electrode. Peaks: HYO = hyodeoxycholic acid; CHOL = cholic acid; CHEN = chenodeoxycholic acid; DEO = deoxycholic acid; LIT = litocholic acid. Sample size, 5 μl.

acids which do not absorb UV-light at 254 nm were analysed in HPLC either with a UV detector operated at 210 nm[12] or a refractive index (RI) detector[13–15]. The present approach offers a new and simple alternative mode of detection.

The conditions imposed by HPLC facilitate the use of detection based on measurements of changes in the double layer capacity. The high value of the solution flow-rate, which increases the rate of transport of a substance to the DME surface due to convection, especially at long drop times[8], enables the determination of lower concentrations than in the similar system under steady-state conditions. The curve of $1/C$ *vs.* E for a solution containing $5 \cdot 10^{-5}$ M of cholic acid (CHOL), and at steady-state conditions was almost indistinguishable from the curve in the absence of CHOL. However, it was possible to detect CHOL in 5 μl of $2.5 \cdot 10^{-4}$ M CHOL injected in the column where it was diluted approximately 400 times by the 1 ml min^{-1} flow-rate. So, the limit of detection of CHOL, determined by the transport rate of the substance to the DME surface, is close to 1 nmol per injection. This value is almost one order of magnitude larger than that for a UV detector operated at 210 nm[12], but still one order of magnitude lower than that for a RI detector[15]. The observed noise level is close to 0.024 μF^{-1}. The linear dynamic range for CHOL determination extends from 1.25 nmol to at least 50 nmol per injection, with a correlation coefficient of 0.998. The sensitivity is close to $8 \cdot 10^4$ sec 1 μF^{-1} mol^{-1} and detector response index is 0.98. The standard deviation of the peak area from nine consecutive injections was 4.5%.

The results obtained enable us to state that a.v. polarographic detection based on the changes in double layer impedance at the DME is a new useful electrochemical method, especially in comparison to other electroanalytical detectors based on measurements of faradaic currents. The limitation of the method can depend on the rate of attaining the adsorption equilibrium at the DME. Also two-dimensional condensation of the adsorbed substance at the DME should be absent; this is normally fulfilled in water–organic solvent mixtures.

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