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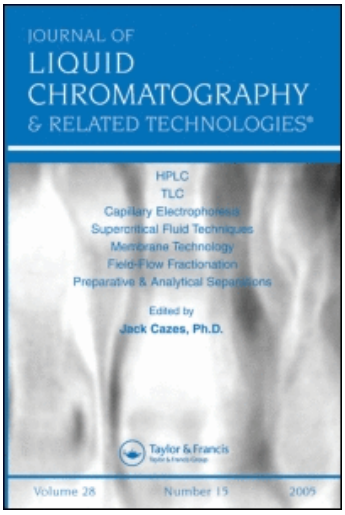

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SURFACTANT MEDIATED GRADIENT ELUTION WITH ELECTROCHEMICAL DETECTION FOR THE ASSAY OF SERUM THYROID HORMONES

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ABSTRACT

Classical HPLC gradient elution is mediated by the programming of pH, ionic strength or organic modifier concentration; however, these components (or factors) affect the electrical double layer of the electrochemical detector. This study demonstrates the use of a surfactant mediated gradient to control the separation of serum thyroid hormones and minimise drift in the background current. It is further demonstrated that the origin of the observed background shift is due to the physical adsorption of the surfactant on the carbon electrode and not to an increase in ionic strength.

Introduction

The amperometric signal, for electrochemical detectors, results from a combination of Faradaic and non-Faradaic processes. The former is caused by the oxidation

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or the reduction of trace electroactive molecules in the mobile phase. The latter, also called "charging current", generally arises from phenomena that take place at the double layer. Since the double layer is similar in nature to a capacitor, changes in the dielectric constant or the conductance of the mobile phase will result in capacitance changes and hence affect the baseline current.

For classical gradients that modulate retention with hydro-organic solvents, pH or ionic strength, trace determinations will be difficult due to large changes in the baseline current. Obviously, gradients that involve small changes in composition will not suffer as much from these baseline problems. However, the real advantage of gradients resides in their ability to cause large changes in adsorption affinity, which allows for concentration, clean-up and separation of molecules with vastly different retention characteristics.

Only a few instances of LC gradient elution with electrochemical detection have been reported in the literature. Two approaches aimed at reducing the baseline problem have been taken, either the detector is modified or the mobile phase components are manipulated.

The reduction of baseline perturbations, through modifications to the electrochemical detector, met with only limited success. The use of a non-ramping differential pulse detector was investigated by Hadjmohammad (1), but

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the base line was less stable than that of an amperometric detector. Tjaden et al. (2) succeeded only in partially correcting for the baseline drift with a dual-cell electrochemical detector, where one of the cells operated at coulometric efficiency, while the other was used to subtract the perturbations. Another proposed strategy allowed the organic-rich eluent to mix with the more aqueous mobile phase in a large volume (35ml) wall-jet detector (3). This proposal assumed that with a large volume electrochemical detector, fluctuations in the mobile-phase composition would not affect the double layer. However, a 20 uA change in the base line was observed with a gradient running between 60% and 100% acetonitrile.

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Variable success was achieved with the manipulation of the mobile phase additives so that minimal perturbation of the signal would be observed. As described above, gradients that produce small changes in retention are of little use, except to reduce the analysis time (4). Typically, successful correction involves the increase of the ionic strength while the organic content is increased (5).

The gradient used in the present study is mediated by the surfactant CAPS (cyclohexylaminopropane sulfonic acid). As shown elsewhere (7,8), the surfactant CAPS is able to cause a large change in retention over a small range of concentrations. This low CAPS concentration should help to avoid any major modification of the parameters that control the background current.

A gradient experiment that takes advantage of the competitive behaviour between the thyroid hormones and the surfactant for sites on the stationary phase can be described in terms of the displacement, from the stationary phase, of the hormones by the CAPS molecule. However, it can be shown (6) that the displacement involved here is different from that described by the technique of "displacement chromatography" (9-14).

Materials and Method

Sample Preparation

The procedure for the analysis of serum thyroid hormones consists of the addition of a 1-ml sample to a disposable borosilicate culture tube (Fisherbrand, cat# 14-962-10B, Fisher Scientific Co., Montreal, Que), followed by the precipitation of the serum protein with 2 ml of methanol (Omnisolv BDH Chemical, Toronto, Ont.). The mixture is mixed and allowed to stand for 5 min. The tubes are centrifuged with a bench top centrifuge for 20 min. The supernatant liquid is transferred, with a disposable pipette, to another disposable borosilicate culture tube. This supernatant liquid is evaporated to dryness with a Speed Vac Concentrator (model SVC-100H, Savant Co., Hicksville, N.Y., 11801) equipped with a refrigerated condensation trap (model RT-100A) and a rotor (model RR40-12). The Speed Vac is a centrifuge in a vacuum chamber kept at constant temperature (45^{o}C). The machine is used to facilitate and speed up the concentration and deposition

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of samples at the bottom of test tubes by evaporation. For the above procedure and serum samples, 14 sample tubes required about 4 hrs to evaporate completely. The evaporated samples were kept in the freezer (-15°).

Stock solutions of hormone were freshly prepared by weighing recently purchased pure crystals of T4 and T3 (Sigma Chemical Co., St.Louis, MO; # T-2877 and T-2376, respectively; stored in a dessicooler at -15°C) in a dark glass volumetric flask and diluting to the mark with methanol. The working standards were prepared by diluting the appropriate volume of stock solution with doubly distilled water. The serum standards of T3 and T4 (Becton Dickinson Immunodiagnostics, Division of Becton Dickinson and Co., Orangeburg, N.Y. 10962; catalog # 611115, lot JN5934, exp. date June 24, 1985 and catalog # 624179, lot KN6084, exp. date Sept.24, 1985 for T3 and T4, respectively), graciously provided by Dr. Solomon of the Royal Victoria Hospital (Montreal, Que.), were stored at 8°C (as recommended by the manufacturer). These serum standards are the same as routinely used to standardize the RIA procedure at the hospital.

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The Chromatographic Analysis

The chromatographic system, thermostated at 50°, and the specific mobile phase preparation have been described elsewere (6-8). The chromatographic medium PRP-1 (Hamilton Co, Reno, NV 89510) was used for both the analytical comlun

(15 cm long and 5 um particle diameter) and the guard column (6 cm long and 10 um particle diameter). The latter was changed when a 1000 psi increase in back pressure was noted. The outlet and the electrochemical detector were insulated with glass wool to minimize heat loss that excessively affected the baseline noise. The electrochemical cell was modified in the following manner: the auxiliary and reference electrode electrical connections were connected together at a platinum disc electrode located inside the thin-layer electrochemical detector on the side opposite from the working electrode. The potential difference between the working electrode and the platinum disc electrode was set to 0.8 Volt.

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The gradient was controlled by a micro computer based system (6, 15). The mobile phase system consisted of: solution "A", a 6.0% acetonitrile and 0.02 molar phosphate solution with a pH adjusted to 11.5 and solution "B", a 0.04 molar CAPS solution made up with solution "A" and adjusted to pH 11.5. The optimum gradient condition consisted of the following conditions and events:

1- Mobile phase is 0% B for 4 min
(go to initial conditions).

2- Inject sample.

3- Clean-up step, mobile phase is 0% B for 6 min.

4- Elution of T3, 10% B for 8 min.

5- Elution of T4, 26% B.

Each sample is solubilized in 0.4 ml of mobile phase "A" before injection. Since each chromatographic run takes

about 25 min., the samples are kept frozen until 20 min before injection.

Results and Discussion

Gradient Design Considerations

The analytical approach, initially considered for the analysis of the hormones, was to allow the unwanted material to elute while the analyte would be retained by the column. Futhermore, during that time their linear speed across the column should be zero to allow for concentration and minimum band width at the inlet of the column. This is crucial since the injection volume is 0.4 ml. The concentration step is possible only with low concentrations of organic modifiers. Preliminary experiments demonstrated that most of the unwanted material was eluted during the first 5 min. (with 0% B). Following this, the hormones need only to be separated. For these reasons, CAPS step gradients were investigated in more detail than others.

For a step gradient, initiated just after the injection of the samples, the retention time is about the same as for an isocratic separation (see table 1), if the final condition of the gradient is the same as the isocratic condition. The isocratic retention volume is systematically slightly lower than the gradient retention volume. This partially results from the delay between the time when the gradient is actuated and the time when the CAPS front

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reaches the column; it is caused by the tube length between the pumps and the column. However, for the purpose of rapid optimization of the component's retention during a gradient, this suffices. The same behaviour is also found for mobile phases with lower concentrations of organic modifiers.

This behaviour would suggest that the equilibrium conditions are established rapidly behind the front of the gradient. This is consistent with the earlier finding of rapid adsorption kinetics for CAPS on the surface of the stationary phase.

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TABLE 1

Retention of thyroid hormones in the isocratic and gradient mode. The experimental conditions are the same as described in the material and method section, where the mobile phase "A" is 0.02 M phosphate, pH 11.5 and 10% acetonitrile and the "B" is 0.03 M CAPS. The flow rate is 2 ml/min. The retention is quoted in mls.

% B	T3		T4	
	Gradient	isocratic	Gradient	isocratic
100	6.0	5.3	10.2	9.5
75	7.5	6.7	12.0	11.0
50	7.5	7.0	13.5	13.5
40	8.7	7.5	15.7	14.5
30	9.5	9.0	17.5	16.5
20	10.5	10.0	21.0	20.5
10	14.0	15.5	30.5	31.0
0		23.0		54.0

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Serum Thyroid Hormones Analysis

The serum standards used here are produced by the manufacturer from pooled serum drawn from volunteers where the native hormone is selectively removed from the pool. The exact method for the removal is considered proprietary (16); however, it can be speculated that the serum is treated with a selective antibody. Using this extracted serum, the nominal amount of hormone is added. From these considerations, it can be assumed that the blank provided is a true blank; it is also of interest to note that a set of aqueous standards produced similar chromatograms as those of the serum based standards. The actual baseline for each analysis was determined from two factors: (i) the constant position (with respect to the wall maximum) of a "notch" in the wall that separates T3 from T4 and (ii) the beginning of the wall maximum (see figure 1). Using all of these considerations the quantitation of each sample was made using this baseline and the peak maximum. The results of the chromatographic analysis of serum T4 are shown in figure 1 and table 2.

Analyte recovery was also assessed in the following manner. Aqueous standards of T4 were recovered with losses up to 15% from 1 ml of water or of physiological buffer (25 mM sodium carbonate, 100 mM sodium chloride adjusted to pH 7.4 with phosphoric acid) or 4% bovine serum albumin in physiological buffer.

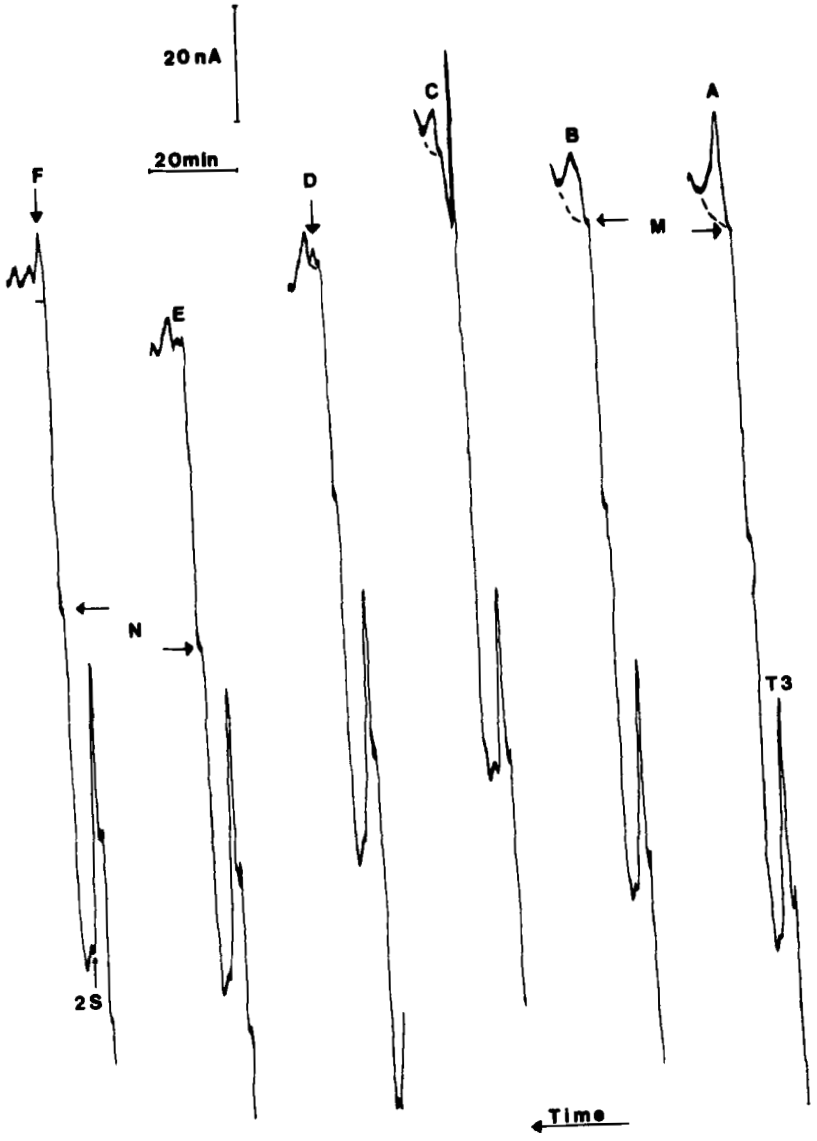

Figure 1. Serum T4 Chromatograms. The serum concentration of T4 are: A, 320; B, 160; C, 80; D, 40; E, 0 and F, 0 with a 160 ng spike, respectively. The base line used for the analysis is also shown. The second gradient step is shown by 2S. The T3 peak is indicated. The notch and the wall maximum that are used to determine the base line for the spiked serum is indicated by N and M, respectively (see text).

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TABLE 2

Signal height for serum-hormone and aqueous hormone sample chromatographic analysis.

amount (ng)	serum signal (nA)	aqueous signal (nA)
480		28.4
320	20.0	20.0
160	12.0	12.0
80	6.4	8.8
40	3.2	
0	1.2	
0 + 160	12.8	

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The determination of serum T3 is difficult because of the low concentration and poor resolution from a large peak. Manipulation of the retention with the concentration of CAPS was not successful since this interference behaves similarly to T3. Also, a large retention increase would have been necessary to reach the required resolution which would have resulted in excessive band broadening. The problem might be solved with a new and more efficient column and by the manipulation of the organic and salt content. From aqueous standards, the detection limit has been evaluated to be 1 ng/injection. However, for sera and in light of the above situation the detection limit has been evaluated to be 4 ng/injection.

Linear regressions

serum	signal = 0.5975 amount + 1.37	corr 0.996
aqueous	signal = 0.4940 amount + 4.454	corr 0.9991

The problem of a large background current shift is dependent, to a very large extent, on the history of the electrode. Recently polished electrodes showed a reduced baseline current shift, up to 10 times less than that shown in figure 1. However, the successive injection of serum based samples increased the baseline current shift at a faster rate than would be observed with aqueous sample. This incrementing baseline current shift eventually reached a maximum. Experimentation with mobile phase "A" and "B" of the same conductance showed that the background current shift is not due to the ionic strength but to the the physical adsorption of the surfactant onto the surface of the electrode. It can be reasonably assumed that the injection of serum based samples would increase the hydrophobicity of the electrode which favors the adsorption of CAPS, leading to an increased baseline current shift with the number of such injections.

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Conclusion

The analytical use of surfactant-mediated gradients with electrochemical detectors was demonstrated to work reasonably well for the analysis of serum thyroid hormones. The background current shift was shown to depend on the adsorption of CAPS on the surface of the electrode, which is also dependent on the history of the electrode.

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