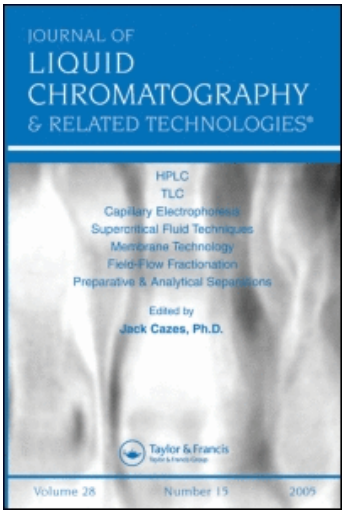

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ELECTROCHEMICAL DETECTION OF RETINOIDS USING NORMAL PHASE HPLC

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ABSTRACT

Non-aqueous electrochemical (EC) detection of 13-cis-retinoic acid, all-trans retinoic, acitretin and vitamin A palmitate in non-aqueous solvents are reported.

Non-aqueous (EC) detection allows for normal-phase chromatography of these compounds prior to detection. The normal phase system used a mobile phase of HEX/THF/AcOH for the separation of all four compounds. The stationary phase was either silica or PVA-sil. The lipophilic salts, t-butylammoniumtetrafluoroborate or t-butyl-ammoniumhexafluorophosphate necessary for EC detection were added post-column.

The limit of detection (LOD) for EC detection of these compounds is approximately 1 ng on column compared with an LOD by UV absorption of 2 ng on column.

The linear detection for these compounds with the EC detector was about two orders of magnitude.

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INTRODUCTION

Electrochemical (EC) detection in HPLC systems has usually been limited to aqueous mobile phases since there is low conductivity in non-aqueous solvents. The EC behavior of retinoids in non-aqueous solvents has been described using cyclic voltammetry (CV) (1-3) but these methods have not been applied to flow through detectors. There have been other reports on the EC detection of retinol and carotenes in a flow through detector, but they have used reversed phase separations and aqueous organic solvents (4-6). The electrooxidation products of retinoids in aqueous solvents have also been investigated (6).

Four selected retinoids (Figure 1) were separated on a normal phase HPLC column using non-aqueous solvents. A lipophilic supporting electrolyte was added post-column to the mobile phase prior to entering the amperometric detector. Hydrodynamic voltammetry of all-trans-retinoic acid (all-trans-RA), 13-cis-retinoic acid (13-cis-RA), acitretin and vitamin A palmitate (vit A pal) was performed using the HPLC/EC system.

EXPERIMENTAL

HPLC Apparatus

The chromatographic system consisted of two HPLC pumps: pump A was a Model 6000A (Waters Chromatography Division, Millipore Corp., Milford, Mass.) and pump B was a Waters Model 6000 with Waters Sapphire Pump Head Retrofit Kit (Swip Precision, Verona, WI). Injector systems were either manual using a 100μl syringe (Hamilton Co., Reno, Nevada) and a Model 7125 (Rheodyne, Cotati, CA) injector equipped with a 50μl loop or automated with a Waters WISP Model 710B. A 100 psi back-pressure regulator (Upchurch, Oak Harbor, WA) was used after the detector cell. The temperature of solvent B was maintained at 40°C, before entering pump B, the solvent was cooled to 32°C using 1 meter of teflon tubing immersed in ice water (Figure 2).

Analytical columns used included a PVA-sil 5μm, (YMC, Morris Plains, NJ), 25cm x 4.6mm id and a 5μm Spherisorb silica, (Universal Scientific, Norcross, GA), 30cm x 3.2mm id, slurry packed in 50:50 methanol/water.

The amperometric detector was a model LC-17A equipped with a 3mm glassy carbon working electrode and a model LC-4B potentiostat (Bioanalytical Systems Inc., West Lafayette, IN). Reference

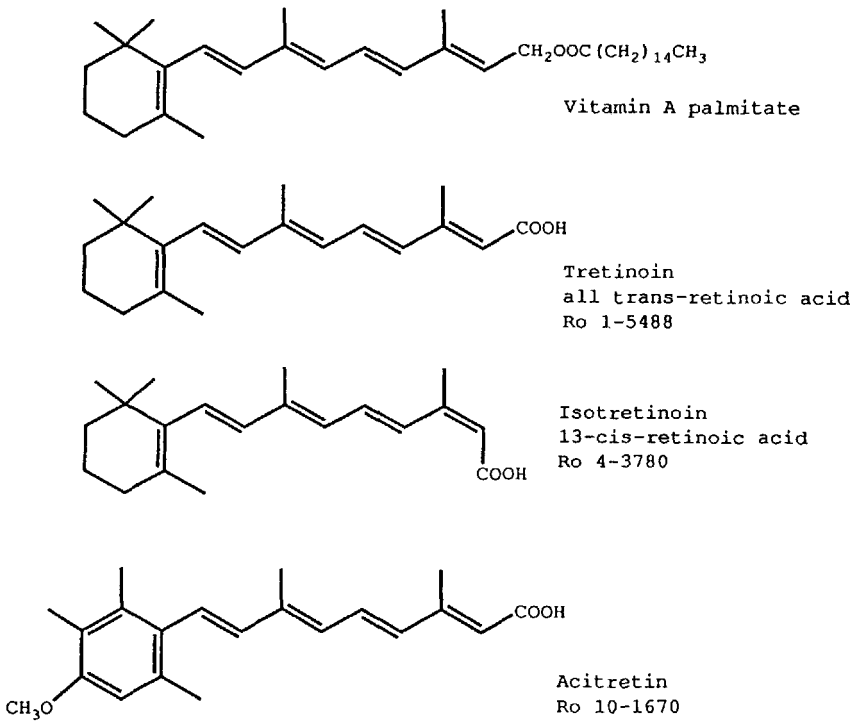

Figure 1 - Structure of all-trans-retinoic acid, 13-cis-retinoic acid, acitretin and vitamin A palmitate.

electrodes used were either a Ag/AgCl filled with aqueous 3.0M NaCl or Ag/AgCl filled with 1.5M $AgNO_3$ in acetonitrile. Ultraviolet (UV) detection was performed with a Model 160 fixed wavelength detector with a 365nm filter (Beckman, Berkley, CA). Chromatograms and data were acquired on a Model 3390A integrator (Hewlett Packard, Palo Alto, CA).

Reagents and Chemicals

All-trans-retinoic acid (Ro 1-5488), 13-cis-retinoic acid (Ro 4-3780) and acitretin (Ro 10-1670) were obtained from Hoffmann-La Roche, Nutley, NJ, and were used without further purification. They were stored at -4°C under argon. Vitamin A

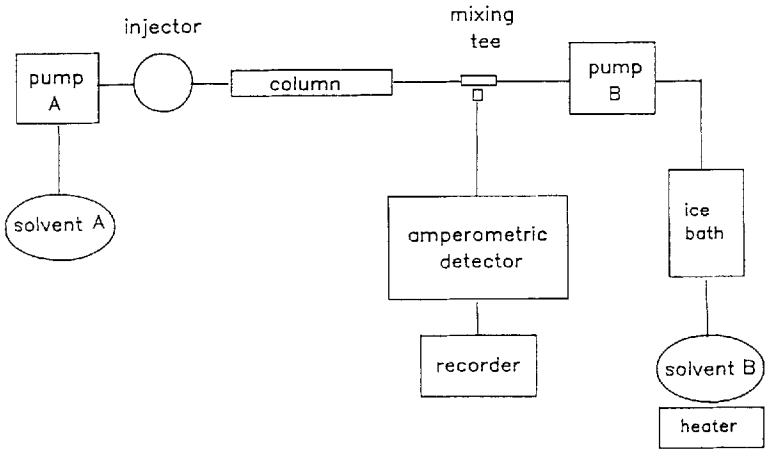

Figure 2 - Apparatus for the electrochemical detection of retinoids with normal phase HPLC.

palmitate (vit A pal), electrochemical grade tetrabutylammonium-tetrafluoroborate (TBATFB) and tetrabutylammonium-hexafluorophosphate (TBAHFP), (Fluka Chemical, Ronkonkoma, NY) were stored in a desiccator at 4°C.

The solvents hexane (HEX), methylene chloride ($MeCl_2$) and tetrahydrofuran (THF), (J.T. Baker Chemical Co., Phillipsburg, NJ) were HPLC grade. THF was stored under nitrogen.

Post-column Electrolyte Concentration

The effect of the post-column electrolyte concentration on the detector response used the apparatus shown in Figure 2. The concentration TBATFB dissolved in $MeCl_2$ (solvent B) was changed while keeping all other parameters constant. Solvent A consisted of 92:8:0.01 HEX/THF/AcOH. The column was a Spherisorb 5μm silica, 30cm x 3.2mm id. The flow rate through both pumps A and B was 1.0ml/min. 50μl of a 1.0μg/ml 13-cis-RA solution in 50:50 HEX/THF was injected for each electrolyte concentration. The retinoids were detected at a potential of +1200mV vs aqueous Ag/AgCl. The concentration of TBATFB in solvent B was varied from 0.3mM to 146mM.

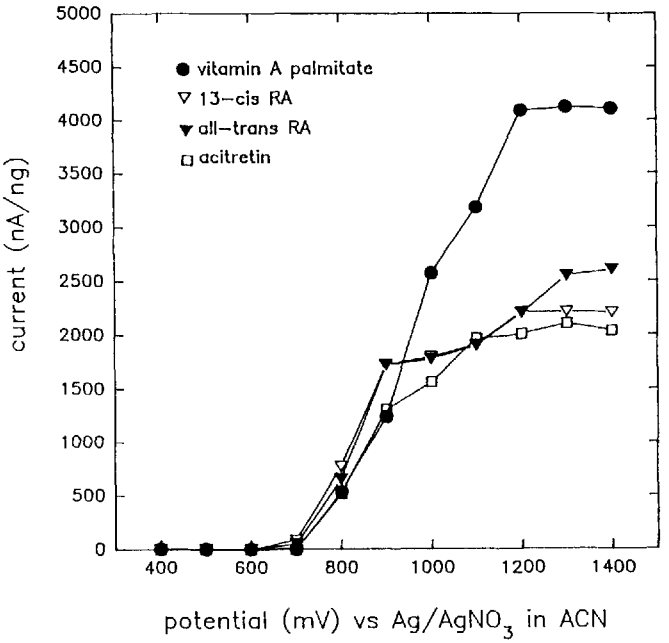

Figure 3 - ECD response with increasing TBATFB concentrations in $MeCl_2$. Pump A - 92:8:0.01 HEX/THF/AcOH, 1.0ml/min. Pump B - $MeCl_2$ with various concentration of TBATFB, 1.0ml/min. Potential - +1350mV vs aqueous Ag/AgCl. Inject 50μg/ml 13-cis-RA.

Hydrodynamic Voltammetry

The hydrodynamic voltammogram (HDV) experiments were performed with the apparatus shown in Figure 2. The potentiostat settings were varied in increments of 50mV from +400mV to +1400mV vs Ag/AgCl in 1.5M $AgNO_3$ in ACN. The flow rate for both pumps A and B was 1.5ml/min. The solvent in pump A was 80:20:0.01 HEX/THF/AcOH (column: YMC-PVA-sil) and pump B solvent was 40mM TBATFB dissolved in either $MeCl_2$ or THF. All solvents were degassed by sonication for 10 minutes.

RESULTS AND DISCUSSION

To optimize the supporting electrolyte solution, the effects of increasing salt in the post-column solvent B were investigated.

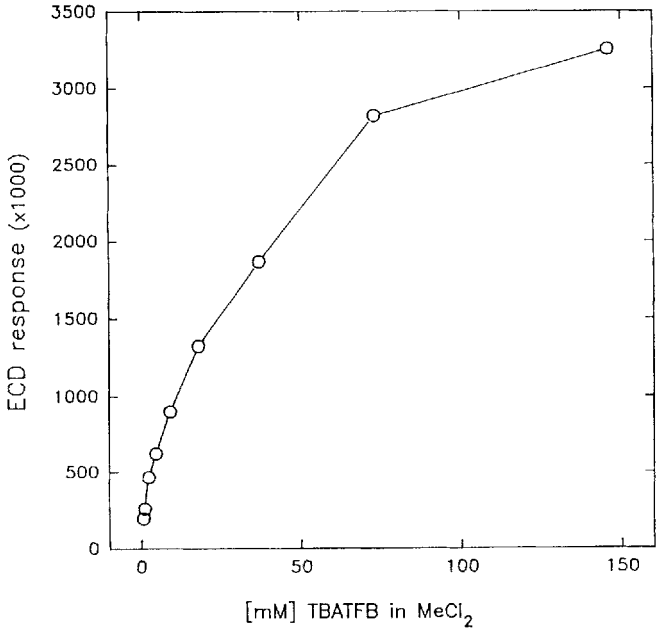

Figure 4 - Hydrodynamic voltammogram of retinoids with 40mM TBATFB in $MeCl_2$ as the post-column electrolyte.

Figure 3 shows the increase in EC response as electrolyte concentration increases. Ideally, a 70mM solvent B electrolyte concentration should be used because further increases in electrolyte concentration do not provide increased detector response. A 40mM TBA salt concentration was chosen for the experiments since concentrations above 40mM caused a precipitation of the electrolyte when mixed with the solvent from the analytical column. The use of higher TBA salt concentrations can be employed if the chromatographic separation allows for a more polar mobile phase. The TBA salts are highly soluble in $MeCl_2$ and THF, but precipitation usually occurred when solvent B is mixed with solvent A. There seemed to be no difference in EC detector response between TBATFB and TBAHFP. Because the TBAHFP salt is less expensive, it has been used for subsequent experiments at the 40mM concentration as the post-column electrolyte.

TABLE 1

Detection Limits of Selected Retinoids[1]

	all-trans retinoic acid[2]	13-cis retinoic acid[2]	acitretin[2]	vitamin A palmitate[3]
LOD non-aqueous EC detector (E=+1200mV)	0.5 ng	0.5 ng	2 ng	2 ng
LOD UV detector (365 nm)	2 ng	2 ng	2 ng	0.7 ng
linearity range non-aqueous EC detector	0.5 ng - 400 ng	0.5 ng - 400 ng	1 ng - 400 ng	2 ng - 4000 ng
correlation coefficient	0.9999	1.0000	0.9999	0.9996
Number of points	4	4	4	4

[1]Signal to noise ratio = 3/1.
[2]Mobile phase, 95:5:0.01 HEX/THF/AcOH, 1.5ml/min, YMC PVA-sil 5μm 25cm x 4.6mm id.
[3]Mobile phase, 97.3, HEX/THF, 1.5ml/min, Spherisorb silica 5μm 30cm x 3.2 mm id.

The HDV's obtained for the retinoids in non-aqueous solvents (Figure 4) closely resembles those reported in aqueous media(4-6). The detector response with $MeCl_2$ as the solvent for the post column electrolyte begins at +700 to +800 mV and reaches a plateau point around +1200 mV. The potentiostat is normally set at +900 mV because higher potential settings lead to large background currents and a decrease in overall signal response. HDV's with TBAHFP in THF were not obtained due to the salt precipitate when the electrolyte solution is mixed with the mobile phase.

The limit of detection (LOD) for the four retinoids in this study are shown in Table 1. The EC detector was linear over two orders of magnitude at a potentiostat setting of +900 mV vs Ag/AgCl in 1.5M $AgNO_3$. The LOD for the EC detector is approximately 1 to 2 times more sensitive than the UV detector except for vit A pal in which case the UV LOD is 3 times that of the EC detector.

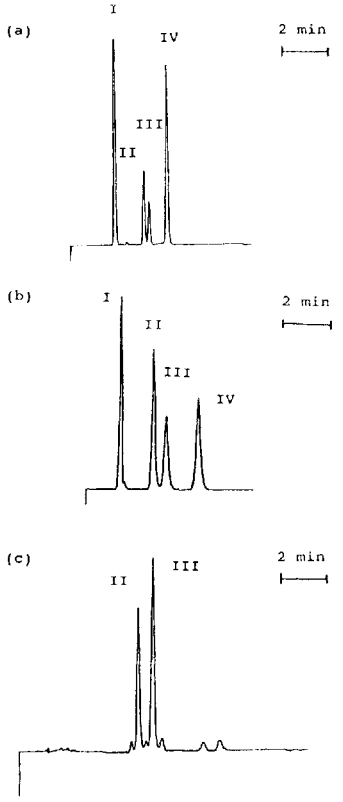

Figure 5 - Chromatography of retinoids, UV detection, 365nm. (a) YMC PVA-sil 25cm x 4.6mm id, 90:10:0.01, HEX/THF/AcOH, 1.5ml/min; (b) 5μm Spherisorb silica 30cm X 3.2mm id, 95:5:0.01 HEX/THF/AcOH, 1.5ml/min; (c) photoisomeric degradation products of all-trans RA, 1μg/ml solution in 50:50 HEX/THF, exposed to white fluorescent lights 48hrs, YMC PVA-sil 25cm X 4.6mm id, 95:5:0.01, HEX/THF/AcOH, 1.5ml/min. (I) vit A pal, (II) 13-cis-RA, (III) all-trans-RA, (IV) acitretin.

Preliminary data obtained with two 10μl stroke volume pumps (Shimadzu LC-600) indicate that detection limits may be lowered with the smaller stroke volume pumps which decrease baseline noise.

Normal phase separation of all four retinoids was achieved on a YMC PVA-sil (25cm x 4.6mm) column with a mobile phase of

90:10:0.01 HEX/THF/AcOH (Figure 5a) or on a 5μm Spherisorb silica (30cm x 3.2mm), and a mobile phase of 95:5:0.01 HEX/THF/AcOH (Figure 5b). The flow rate was 1.5ml/min. Figure 5c shows the separation of suspected geometric photoisomers of a 1μg/ml all trans RA solution in 50:50 HEX/THF after it was exposed to white fluorescent light at ambient temperatures for 48 hours. The YMC PVA-sil column with a mobile phase consisting of 95:5:0.01 HEX/THF/AcOH was used for this separation. If the mobile phase used to separate the retinoids contained less than 92% hexane, then baseline separation between all-trans-RA and 13-cis-RA could not be achieved using the silica column. For baseline separation between the all-trans and 13-cis isomers of retinoic acid separated on the PVA-sil column required a minimum of 85% hexane in the mobile phase. Vit A pal is more non-polar than the retinoic acids, and eluted close to the solvent front. Therefore a mobile phase with high hexane content was not suitable for vit A pal analysis. Acitretin in the above mobile phase does not elute in the area of the chromatogram where the geometric isomers of retinoic acid elute, it could be used as an internal standard for the quantitation of all-trans or 13-cis retinoic acid mixtures.

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