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Separation of neutral lipids by high-performance liquid chromatography: quantification by ultraviolet, light scattering and fluorescence detection

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Abstract

The recent increased use of cell cultures to model physiological events, in particular signal transduction and traumatic injury, has produced a need for a quantitative, high-performance liquid chromatographic separation of neutral lipid classes with a high degree of resolution and reproducibility. We report an isocratic separation using a Phenomenex Selectosil silica column (5 μm). Two solvents were used, 1.2% 2-propanol in *n*-hexane containing 0.1% acetic acid (90%) and *n*-hexane (10%) at a flow-rate of 0.6 ml/min. Column temperature was maintained at 55°C and this temperature was critical for baseline resolution of 1,3-diacylglycerol and cholesterol. The use of 10% *n*-hexane permitted the resolution of low polarity compounds such as butylated hydroxytoluene, triacylglycerols and cholesteryl esters. All of the detectors used produced standard curves with linearity over a wide concentration range.

Keywords: Lipids; Neutral lipids

1. Introduction

Previous neutral lipid separations routinely relied upon thin-layer chromatography (TLC) techniques to separate neutral lipid fractions from tissue lipid extracts [1,2]. While solid-phase extraction has been used by our lab [3] and others [4] to fractionate samples, these methods are time consuming and often require further separation and analysis. However, high-performance liquid chromatography (HPLC) has been used to separate neutral lipids using several different column types and solvent systems, including but not limited to: cyanopropyl [5,6]; reversed-phase [7,8] and silica [9–15] columns. Several of these solvent systems are incompatible with low wavelength ultraviolet (UV) detection [9–12], although others are compatible with low wavelength UV detection [5,7–9,13,14]. Numerous detection systems are used to quantify neutral lipids including: flame ionization [9,10,16]; evaporative light scattering [11,12,15] and UV light absorbtion [6,7] detectors.

The increased use of cell culture models has resulted in the need for a sensitive, reliable HPLC separation to fractionate the major neutral lipid classes from small samples sizes. Here, we report the first use of a HPLC separation designed to utilize up

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to three different modes of detection to separate and quantify neutral lipids from cell culture size samples.

2. Experimental

All solvents were HPLC grade and were purchased from E.M. Science (Cherry Hill, NJ, USA). Trioleylglycerol, 1,2-dioleylglycerol, 1,3-dioleylglycerol, cholesteryl oleate and free fatty acids were purchased from NuChek Prep (Elysian, MN, USA). α-Tocopherol was purchased from Kodak (Rochester, NY, USA). Cholesterol was purchased from Steraloids (Wilton, NH, USA). Cell culture neutral lipid fraction was from rat cortical astrocytes in culture for 28 days and subjected to 12 h of oxygen–glucose deprivation.

2.1. Instrumentation

The HPLC system consisted of a Beckman 420/421 controller (Fullerton, CA, USA), two Beckman 114 M pumps, a Beckman 210 A injector, an ISCO V_4 variable-wavelength UV–Vis detector (Lincoln, NE, USA), a Shimadzu RS-535 fluorescence detector (Kyoto, Japan), a Varex ELSD-II evaporative light scattering detector (ELSD) (Burtonsville, MD, USA) and a Jones Chromatography heating block (Littleton, CO, USA). The column used was a Phenomenex Selectosil silica column (250×4.6 mm, 5–6 μm, Torrance, CA, USA).

2.2. Sample preparation

The neutral lipid and phospholipid fractions were separated using HPLC or silicic acid solid-phase extraction. Prior to HPLC analysis, the neutral lipid fractions were filtered through a 0.2-μm Nylon filter (Rainin, Woburn, MA, USA), evaporated to dryness under a stream of nitrogen, dissolved in *n*-hexane–2-propanol–acetic acid (98.7:1.2:0.1, v/v) and transferred to a limited volume vial. Just before HPLC, the samples were evaporated under a stream of nitrogen and redissolved in a known amount of *n*-hexane–2-propanol–acetic acid (98.7:1.2:0.1, v/v). The samples were injected into a 20-μl sample loop.

2.3. Separation

This HPLC method used a two solvent system. Solvent A was 1.2% 2-propanol in *n*-hexane containing 0.1% acetic acid. Solvent B was *n*-hexane. Optimal proportions of solvents A–B were 90:10 with an optimal flow-rate of 0.6 ml/min.

The UV and fluorescent detectors were set up in line to continuously monitor the eluent. The absorbance of the eluent was constantly monitored at 205 nm and the fluorescence detector was set at an excitation wave length of 295 nm and emission monitored at 340 nm. Detector response was monitored using a BD-41 Kipp and Zonen (Deventer, Netherlands) strip chart recorder or an ISCO recorder. For quantitative analysis, peak area data were collected by a Nelson Analytical 760 series intelligent interface (Cupertino, CA, USA) and converted from analog to digital data. Peak areas were calculated using Nelson Analytical 2600 software.

The ELSD was hooked up in line with the UV and fluorescence detectors. Because of the inability to recover the sample using an ELSD, this detector was placed last in the series of detectors. A stream-splitting option can be used to divert up to 50% of the eluent from the detector, permitting collection of fractions for further analysis. The ELSD conditions were inlet temperature, 106°C, and a nitrogen carrier gas flow-rate of 35 l/min.

3. Results

3.1. Effects of column temperature

The effect of column temperature is seen in Fig. 1. Cholesterol and 1,3-dioleylglycerols elute with extremely close retention times using the Selectosil silica column and this solvent system. In an effort to completely resolve these two neutral lipid components we altered column temperature. As seen in Fig. 1, with increasing temperature there is an increase in separation between these two compounds. At a column temperature of 50°C, there was complete baseline resolution. The temperature effect may be a result of different adsorption and desorption characteristics of cholesterol and 1,3-dioleylglycerols, causing altered interaction with the silica column.

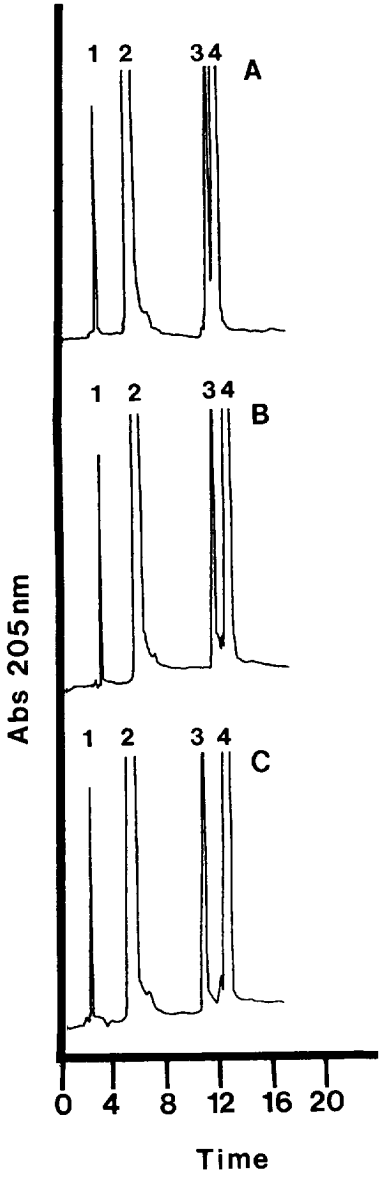

Fig. 1. Column temperature effects on resolution of cholesterol and 1,3-dioleylglycerols. For all panels, flow-rate was 1.5 ml/min and the solvent was *n*-hexane–2-propanol–acetic acid (98.7:1.2:0.1, v/v). (A) Column temperature was 40°C, note only the partial resolution of the two compounds (compounds are nearly unresolvable at 35°C). (B) Column temperature was 45°C, note near baseline resolution of the two compounds. (C) Column temperature was 50°C, note baseline resolution of the two compounds. For all panels the peak assignments are as follow: 1=trioleylglycerol; 2=free fatty acids; 3=cholesterol and 4=1,3-dioleylglycerol.

3.2. Effect of flow-rate

The effect of flow-rate on cholesterol and 1,3-dioleylglycerol resolution is seen in Fig. 2. With the column temperature held constant at 45°C, the flow-rate was varied between 0.8 ml/min to 1.2 ml/min. Unlike in Fig. 1, there were nearly equal amounts of cholesterol and 1,3-dioleylglycerol present relative to each compound's molar extinction coefficient. As the flow-rate was decreased, there was a concomitant increase in retention times. This resulted in the partial resolution of cholesterol and 1,3-dioleylglycerol at a flow-rate of 1.0 ml/min (Fig. 2, panel B) and at a flow-rate of 0.8 ml/min (Fig. 2, panel C). However, unlike temperature, this alteration in flow-rate did not result in a baseline separation of these two compounds.

3.3. Separation of cell culture size sample

A normal separation of a cell culture size samples is shown in Fig. 3 using the following parameters: flow-rate 0.6 ml/min; column temperature 55°C and a *n*-hexane proportion of 10%. In Fig. 3 (upper panel), a UV absorption tracing of a neutral lipid fraction from an astrocyte cell culture is shown. The large fatty acid peak was a result of elevated fatty acids following oxygen–glucose deprivation for 12 h. Note the near baseline resolution of butylated hydroxytoluene, triacylglycerol, and cholesteryl esters. Cholesterol was well resolved, although no 1,3- or 1,2-diacylglycerols were evident. The detector output to the recorder was set at 0.1 AUFS. In Fig. 3 (lower panel), the fluorescence tracing is seen with the resolution of α-tocopherol. The butylated hydroxytoluene gave a strong fluorescence signal as does an unidentified compound that elutes just prior to free fatty acids.

3.4. Quantification

This method permits the direct quantification of several neutral lipids by analysis of peak area. Through the use of multiple detectors, several different compounds can be quantified during this separation. Fluorescence detection was used to quantify α-tocopherol, with a linear range between 252 pg and 2.5 ng. The intra-day variability was between

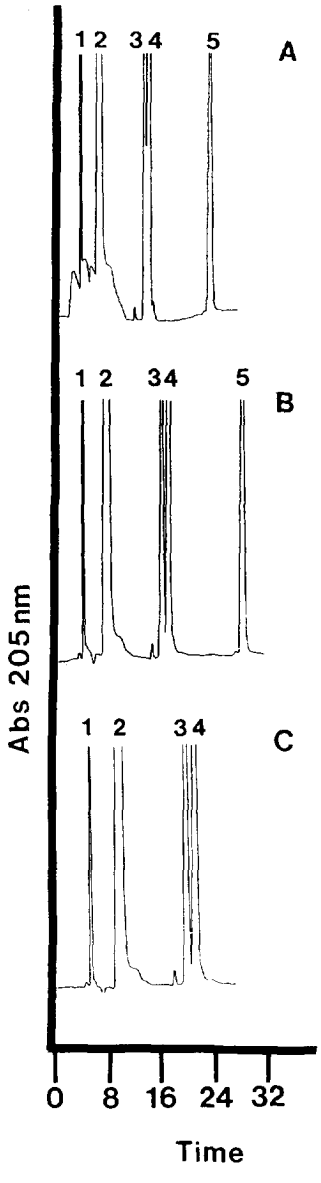

Fig. 2. Flow-rate effects on resolution of cholesterol and 1,3-dioleylglycerols. For all panels, column temperature was maintained at 45°C and the solvent was *n*-hexane–2-propanol–acetic acid (98.7:1.2:0.1, v/v). (A) Flow-rate was 1.2 ml/min; note the incomplete resolution of the two compounds. (B) Flow-rate was 1.0 ml/min; note the partial resolution of the two compounds with an increase in retention time. (C) Flow-rate was 0.8 ml/min; note the partial resolution of the two compounds with an increase in retention time. For all panels the peak assignments are as follow: 1=trioleylglycerol; 2=free fatty acids; 3=cholesterol; 4=1,3-dioleylglycerol and 5=1,2-dioleylglycerol.

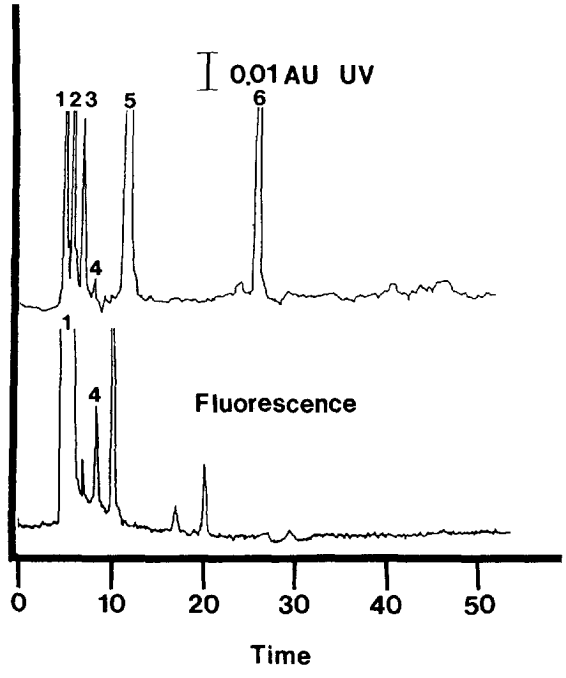

Fig. 3. Separation of an astrocyte cell culture neutral lipid fraction. Column temperature was 55°C and flow-rate was 0.6 ml/min. Solvent composition was *n*-hexane–2-propanol–acetic acid (98.7:1.2:0.1, v/v) mixed with *n*-hexane for a final proportion of 90:10. Modifying the solvent with 10% *n*-hexane permitted the resolution of butylated hydroxytoluene, triacylglycerols and cholesteryl esters to near baseline. The large free fatty acid peak is the result of cell culture exposure to 12 h of ischemic-like conditions. The fluorescence spectrum shows the strong emission peak resulting from butylated hydroxytoluene. α-Tocopherol is resolved from both butylated hydroxytoluene and an unidentified peak eluting at approximately 10.5 min. For all panels, the peak assignments are as follows: 1=butylated hydroxytoluene; 2=triacylglycerol; 3=cholesteryl esters; 4=α-tocopherol; 5=free fatty acids and 6=cholesterol.

1.3–4.6% depending upon standard concentration. The fluorescence detector parameters were excitation at 295 nm with an emission of 340 nm.

Cholesterol can be quantified using either absorbance at 205 nm or the ELSD [17]. Our results indicate that both instruments gave a linear standard curve, although there were two different linear ranges. UV absorbance was linear between 0.8 to 75 μg, although recently using a Shimadzu SPD-10A UV absorbance detector (Kyoto, Japan), levels to 40 ng were detectable, with an intra- and inter-day variability between 0.8–4.4 and 0.7–6.2% respec-

tively, depending upon standard concentration. The linear detectable range for the Varex ELSD was 2–150 μg.

Cholesteryl esters and free fatty acids can be quantified using the Varex ELSD. Both of these lipids are difficult to quantitate using absorbance at 205 nm because of the varying degree of unsaturation [18]. Using the ELSD, this problem is overcome because the degree of unsaturation does not appreciably effect the amount of light scattered [19–21]. The linear detectable range for free fatty acids was 50–800 μg, although samples could be reliably measured at levels exceeding 10 μg. The intra-day variability was between 1.6–4.4% depending upon standard concentration. For the fatty acids, a commercially prepared fatty acid mixture with a varying degree of unsaturation was used as a standard. For cholesteryl oleate, the detectable range was 6–100 μg. The intra-day variability was between 1.6–3.1% depending upon standard concentration.

4. Discussion

With the increased use of cell cultures to model in vivo events, a method for the separation and quantitation of neutral lipids combined with direct quantitation of many of these lipid classes was needed. While conventional TLC procedures have been used with tissue samples [1,22] as well as cell cultures samples [23], these methods are often susceptible to problems because of varying solvent mixtures, temperature and, more importantly, humidity. Day to day variability in R_F and resolution is a common occurrence. While TLC methods offer speed, direct quantification of the bands is difficult without a densitometer. Furthermore, densitometry is limited in sensitivity and is subject to a greater degree of variability compared to the detection methods used here. Through the use of the HPLC method presented here, many of the above mentioned problems are avoided.

The method presented herein allows direct quantification of the major neutral lipid classes using one or more detectors. Our experience has shown that the method was very useful in studying *cis*-parinaric acid incorporation into the neutral lipids of L-cell fibroblasts [24] and for altered lipid metabolism following combined oxygen–glucose deprivation in astrocytes [25]. Unlike TLC procedures, cholesterol was directly quantitated by absorbance at 205 nm, although cholesterol can be measured using gas–liquid chromatography (GLC) [26]. However, the use of GLC to measure a mixture of neutral lipids has not been reported. GLC has been used to determine triacylglycerol molecular species [27] and diacylglycerol molecular species [28], however, there are no reports that suggest that the separation of complex mixture of neutral lipids of varying polarity is possible [29].

Using the Varex ELSD, the levels of triacylglycerols, cholesteryl esters, cholesterol and in some instances free fatty acids can be quantified from cell culture size samples. Samples containing a large amount of fatty acids are more suited for quantification using the Varex detector for fatty acid analysis. During ischemic conditions in culture, some cell culture samples reach the 50–70 μg of total fatty acids, enough for adequate detection using the Varex ELSD. Furthermore, fatty acid detection at concentrations of 10 μg may be possible, however the major drawback of ELSD is the overall low sensitivity and its inherent destructive nature. Although fatty acids cannot be measured by absorbance at 205 nm, a qualitative assessment can be made and the free fatty acid fraction collected, methylated and quantitated in the low ng range using GLC [25].

We found temperature was a useful tool for separating 1,3-diacylglycerol and cholesterol. By elevating the column temperature, these two compounds were completely resolved. Solvent polarity alterations had a similar effect but only for lipids with a polarity equal to or less than free fatty acids. Modifying the solvent A with 10% *n*-hexane permitted the resolution of butylated hydroxytoluene, triacylglycerols and cholesteryl esters to near baseline. In our laboratory, the same column has been used for over 400 h of separations with no extensive loss of resolution. Hence, this method provides an excellent means to separate cell culture size neutral lipid samples in a quantitative mode.

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