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CONTINUOUS-FLOW SCANNING OF SELECTED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY PEAK COMPONENTS BY MICROPROCESSOR CONTROL

APPLICATION TO ANALYSIS OF EXTRACTS FROM HUMAN LYMPHOCYTES

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SUMMARY

Continuous-flow wavelength scanning of compounds separated by high-performance liquid chromatography is achieved through the use of fixed and variable-wavelength micro ultraviolet detectors connected in series but separated by a low-pressure three-way valve. Activation of the valve allows entrapment of selected peaks in the variable-wavelength detector without interfering with the response of the fixed-wavelength detector which is utilized for peak quantitation. A microprocessor program is employed to maintain control and accuracy during the scanning sequence. Good correlation was found between ultraviolet spectra of standards obtained on a conventional spectrometer and those on separated peaks. This system allows the identification and quantification of picomole amounts of peaks separated during one analysis of a biological sample.

INTRODUCTION

The high sensitivity and resolution of complex mixtures of biological origin by high-performance liquid chromatography (HPLC) generates a problem in the identification of separated compounds. Most methods of chemical analysis, such as nuclear magnetic resonance or infrared spectroscopy, or elemental analysis, require large sample sizes (*i.e.*, milligram amounts), amounts that are far above separations obtainable by analytical HPLC. The use of mass spectroscopy, while highly sensitive for sample identification, requires a volatile sample. Comparison of retention time(s) with known standards is useful, but not sufficient by itself as an absolute means of identification. Compounds with similar chromatographic properties may co-elute, and precise identification requires the comparison of retention times under different chromatographic conditions. Thus, for most compounds of biological interest, the

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use of spectroscopic characterization provides a simple on-line means of HPLC recognition. Absorbance ratios have been widely used as an aid in peak identification[1–6]; however, optimal utilization requires prior knowledge of the absorbance properties of the components in the mixture which is being separated. For samples of biological origin, the exact nature of the individual compounds is not always known. The more refined technique of stopped-flow scanning allows examination of the entire spectrum of a chromatographic peak using a variable-wavelength HPLC detector[5,7,8]. While this method is highly useful when examining an unknown compound, a major drawback is the requirement to stop chromatographic flow during the duration of the wavelength scan. The start/stop of flow makes peak quantitation unreliable and requires a second injection for this purpose[9]. To aid in the combined identification–quantification when limited amounts of material are available for analysis, a method is described below which allows for peak scanning(s) during continuous chromatographic flow to one of two detectors connected in tandem to the outflow of a HPLC column. Microprocessor control is utilized to provide an automatic scanning sequence. The application of this technique will be shown for the analysis of an extract of human lymphocytes applied to a strong anion exchanger microparticulate HPLC column.

METHODS

Apparatus

A Spectra Physics 8000A liquid chromatograph (Santa Clara, CA, U.S.A.) equipped with a two-channel data system was used for all separations. On-line detection was performed using a Spectra Physics Model 8310 fixed-wavelength (254 nm) detector and a Schoeffel Model 770 spectroflow variable-wavelength detector equipped with SFA 339 wavelength drive and MM 700 Memory Module (Kratos, Westwood, NJ, U.S.A.). A low-pressure three-way valve (No. 1-43-900, General Valve Corp., East Hanover, NJ, U.S.A.) was connected between the two detectors to divert the flow during the peak trapping in the variable-wavelength detector. The output of the variable-wavelength detector was connected to a Houston single-channel Omniscribe recorder equipped with an event marker and remote control switch for the chart speed (Houston Instrument Co., Austin, TX, U.S.A.), and to an analogue-to-digital converter (Spectra Physics). Spectra from standards were obtained on a Beckman Acta II spectrophotometer at a scan rate of 100 nm/min. Optoisolators were employed to serve as an electronic buffer between the relays activating the various events during the wavelength scanning sequence and microprocessor controlled switches (Fig. 1). A constant voltage d.c. power supply was used for this circuit. A detailed schematic is shown in Fig. 2. Electronic components were obtained from Radio Shack.

Chemicals and supplies

The mobile phase was prepared from reagent grade monobasic potassium phosphate (Sigma, St. Louis, MO, U.S.A.) and potassium chloride (Mallinkrodt, Louisville, KY, U.S.A. (the phosphate was purified according to the method of Shmukler[10]. The water was double-distilled and deionized. Prior to use, the buffers were filtered through a 0.2-μm filter (Sartorius, Haywood, CA, U.S.A.). Degassing was

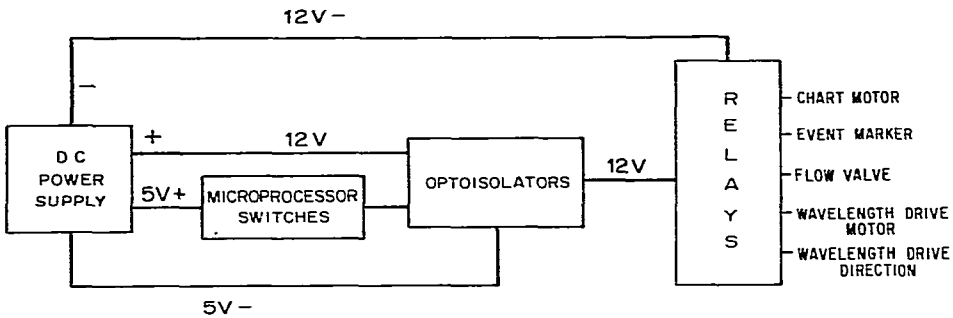

Fig. 1. Accessory control unit of microprocessor controlled continuous-flow peak scanning. A regulated d.c. power supply is utilized to provide 12 V for the relays and 5 V for the optoisolator microprocessor switching circuitry. The activation of the various relays whose control function is listed above is achieved via a two-step switching process: the microprocessor switch applies power to an optoisolator which in turn provides power to a given relay.

achieved via a helium purging system (Spectra Physics). Ribonucleotide standards were obtained from Sigma.

Chromatographic columns

A Whatman Partisil 10 SAX (25 × 0.46 cm I.D.) column was used for analytical separation (Whatman, Clifton, NJ, U.S.A.). A guard column (7 × 0.2 cm I.D.) filled with Pellicular Anion Exchanger (Whatman) was used to protect the analytical column. A precolumn (25 × 0.46 cm I.D.) filled with 37–53-μm silica (Whatman) was utilized to saturate the mobile phase with dissolved silica and served to extend the lifetime of the analytical column.

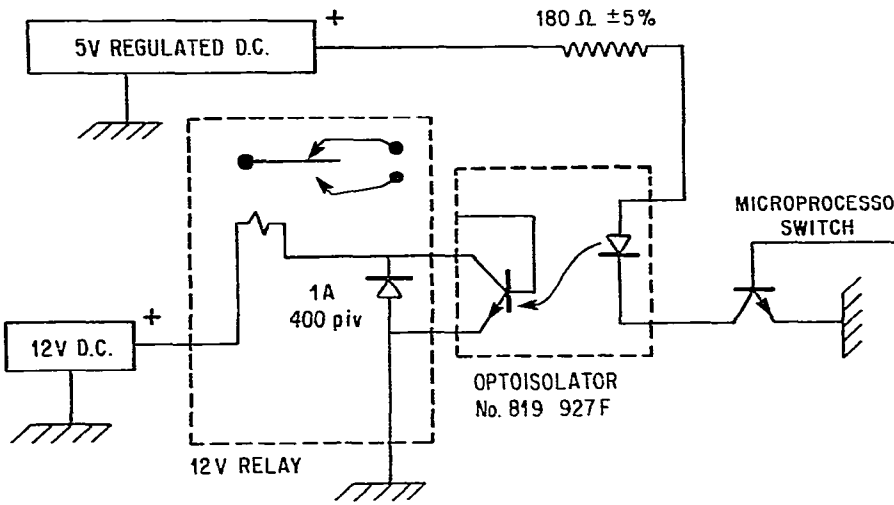

Fig. 2. Electronic circuitry of microprocessor–optoisolator 12-V relay switch. The closing of the normally open state of a microprocessor switch provides ground to the optoisolator circuitry allowing the 12 V d.c. to flow to the control relay. This three-step process allows for a rapid switching sequence as well as for protection of the microprocessor from back power surges from the relay coil.

Biological material

Informed consent for obtaining peripheral blood from donors was according to the provisions of the Helsinki Conference. Lymphocytes were purified as previously described[11]. Cell pellets containing 2–30 × 10^6 cells were extracted using 60% methanol essentially as reported by Donofrio *et al.*[12]. For each 10^7 cells, 200 μl of 60% methanol were added for extraction of the cell pellet. Extracts were taken to dryness using a Savant Speed Vac Concentrator (Savant, Hicksville, NY, U.S.A.), hydrated with water, and stored frozen at −70°C until subjected to HPLC analysis.

Chromatographic conditions

The separation conditions were a modification of the method of McKeag and Brown[13]. The elution conditions were as follows: temperature, 40°C; flow-rate 1.5 ml/min from 0 to 40 min, 3.0 ml/min from 40 to 52 min, 2 ml/min from 52 to 72 min; buffers, (A) 0.007 *M* KH_2PO_4, 0.007 *M* KCl, pH 4.0, (B) 0.25 *M* KH_2PO_4, 0.5 *M* KCl, pH 5.0; mobile phase, 0–5 min isocratic A, 5–40 min linear gradient A → B; 40–52 min isocratic B, 52–67 min linear B → A, 67–72 min isocratic A.

HPLC ultraviolet scanning

Ultraviolet (UV) spectra of trapped peaks were obtained at high sensitivity ranges of the Model 770 variable-wavelength detector (0.04–0.01 a.uf.s.). To allow distortion free spectra, a background spectrum was stored using buffer A on the 0.1 a.u. memory range of the MM 700 background subtract accessory. Since the steps followed for preparation of the mobile phase resulted in no significant difference in the absorbance properties of buffers A and B, buffer A was used as the blank for all spectral scans. Spectra were stored/scanned at a rate of 100 nm/min.

Program operation

The subroutine controlling the operation of the function relays is a simple eleven-step procedure that can easily be adapted to any microprocessor system which can be programmed and which contains switched on/off transistors. The flow chart of this program is contained in Fig. 3.

RESULTS

Fig. 4 shows a flow diagram of the chromatographic system employed for continuous-flow scanning. Prior to the trapping of a chromatographic peak, the outflow from the analytical column passes through detector 1 (for quantitation at 254 nm) to detector 2 and out to waste. For UV scanning of a selected peak, the detector response is followed either by the pen deflection of recorder 2 or by the digital signal on the data channel. Upon observation of the top of the peak, activation of the three-way valve diverts the flow from detector 2 to waste. The 1-m height of the detector 2 waste outlet prevents diffusion outflow of the trapped peak. Once the desired beginning wavelength is set, the scanning program sequences shown in the flow chart in Fig. 3 are started via a microprocessor command. A typical wavelength scan sequence from 215 to 350 nm takes *ca.* 1.5 min. Duplicate or triplicate scans showed identical patterns (data not shown). Flow to the second detector is re-established when power is removed from the flow valve. No pertubation to detector 1 was

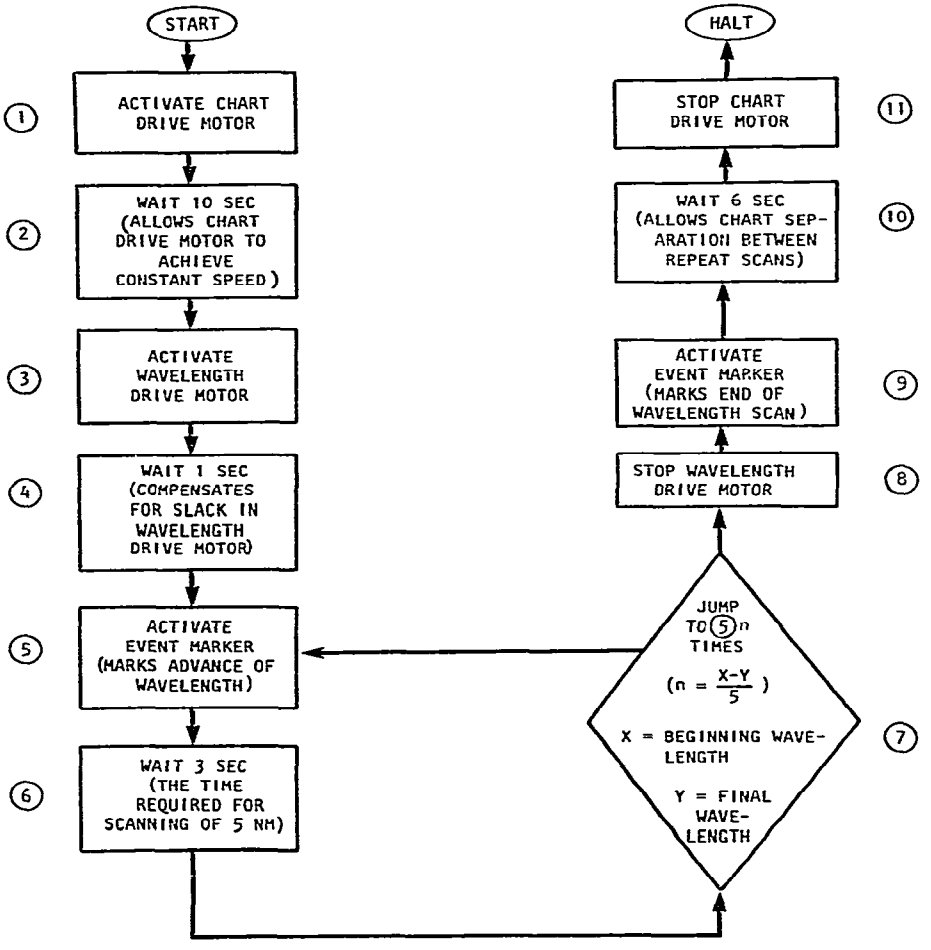

Fig. 3. Flow chart of program controlling automatic scanning sequence with wavelength notation.

observed upon the activation of the three-way valve. A new peak trapping and scan sequence could be begun once a new baseline was established.

Fig. 5 shows an elution profile obtained from an extract of human lymphocytes. The peak identifications were based on retention times of standards and verified by wavelength scanning and 280/254 nm ratios. Selected peaks sequentially scanned in the UV region during the course of one chromatographic separation are shown in Fig. 6 with spectral scans of reference standards. With the exception of the far-UV portion of the spectra, where differential O_2 absorption and variations in the monochromaters of the two instruments are more apparent, the agreement between scans is evident. The data obtained with IMP (Figs. 5 and 6B) show that closely resolved peaks can be subjected to continuous-flow UV scanning with satisfactory results.

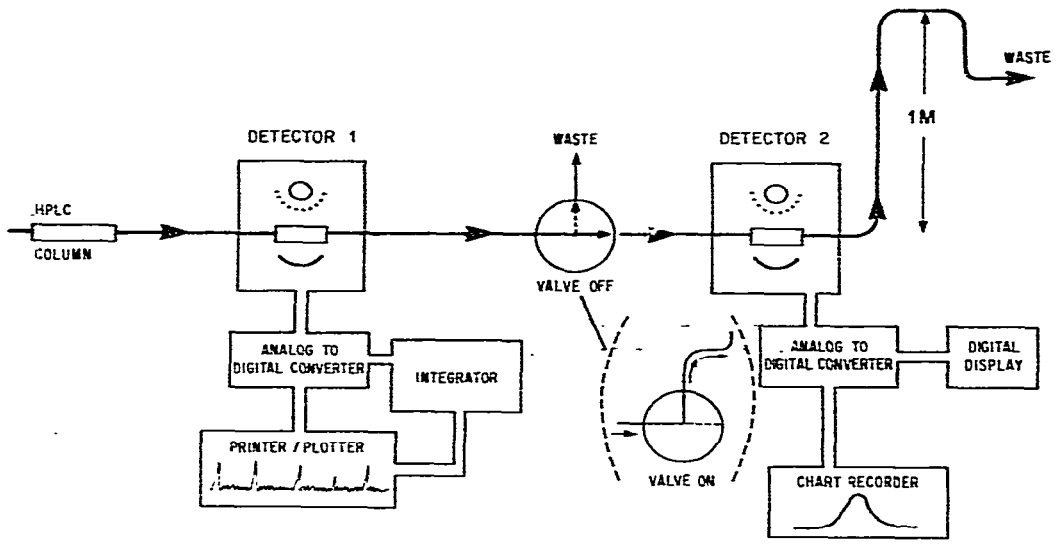

Fig. 4. Post-column elution flow diagram. The flow from an analytical HPLC column is allowed to sequentially pass through two detectors. The three-way valve between detector 1 and detector 2, in its inactivated state, allows the flow to pass directly to detector 2, and out through a 1-m high waste outlet. When current (110 vac) is applied to the valve, flow is diverted from detector 2 to waste leaving material trapped in the second detector. The 1-m height of the waste outlet of detector 2 coupled with the "on" configuration of the three-way valve prevents any diffusion of the trapped sample out of detector 2.

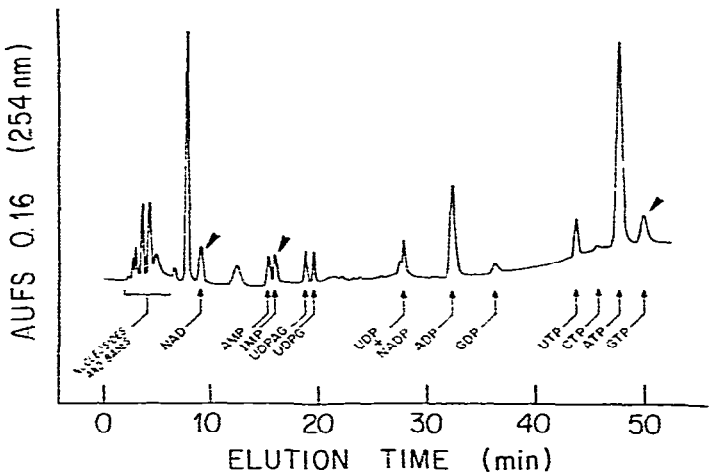

Fig. 5. Separation of nucleotides on Partisil 10 SAX from an extract prepared from 3×10^6 human lymphocytes. Chromatographic conditions are as described in the Methods. The marked peaks were subjected to peak trapping in the variable-wavelength detector during the same chromatographic separation.

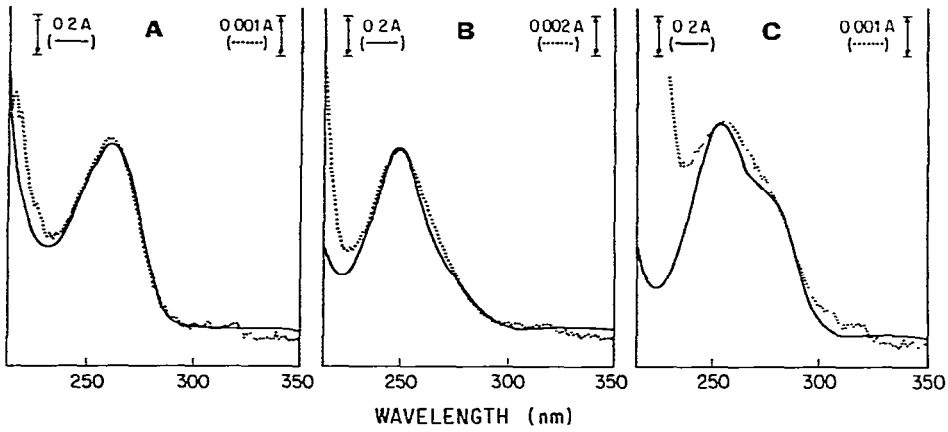

Fig. 6. UV wavelength scans of trapped HPLC peaks by the continuous-flow scanning technique (...) and reference compounds (—) by a standard UV spectrometer. A = NAD; B = IMP; C = GTP. Scanning range, from 215–350 nm; absorbance range as indicated.

DISCUSSION

A method is described which allows for the multiple use of HPLC separations where quantitation and peak identification by wavelength scanning on the same sample can be achieved. This technique, which makes use of two detectors, results in no interference in the chromatography as can result with stopflow scanning, and is of value particularly when limited material is available for analysis. Previous work has demonstrated the applicability of stopped-flow wavelength scanning to aid in the identification of a variety of components separated by HPLC[5,7,8,14]. A method for simultaneous multiwavelength detection has been described, but its utilization is limited by the cost of a rapid scan spectrometer and the complexity of the data processing and control systems[15]. The system employed in this study allows flexibility in the number of scans obtainable and the wavelength range of the scan, and is simple to set up.

Microprocessor control was utilized to carry out a repeatable sequence throughout the spectral scan along with user-supplied commands for peak trapping and initiation of the UV scan sequence. While a fully automated UV scan sequence was not carried out, owing to minor variations in retention times that occur with an ion exchange column, the consistent separations obtainable with a reversed-phase column would be ideally suited for this purpose. Conversely, trapped peak scans via manual control are possible, although considerable sacrifice in the consistency and reproducibility of the recorded spectra results. The system described in this paper was developed around the capabilities of a liquid chromatograph with a self-contained microprocessor having the ability of accepting a program controlling transistorized switching. The program and electronics detailed in the Figs. 1–3 can easily be adapted to any of a number of microprocessor controllers and data systems that are currently available for connection to an existing HPLC system (Spectra Physics SP4000, SP4100, Digital MINC 11, Waters Model 720 Controller). The use of a separate regulated power supply was required to assure the reproducibility in the operation of the transistorized switching circuits.

The ability to trap a peak in the small volume employed by most commercial HPLC detectors (*ca.* 10 μl) in a pathlength of 0.5–1 cm allows a sensitivity as low as 100 pmoles for wavelength characterization on typical compounds (*e.g.*, with $E_{mm} \approx 10$). The equivalent characterization using the cell configurations and sensitivity available with regular UV spectrometers on an HPLC separated component, could only be attempted with material that is collected from preparative scale HPLC separations.

The examination of purified preparations from samples of biological origin often leads to small yields of material for HPLC analysis as a result of the purification process. While not as detailed as other spectroscopic measurements, UV spectra do provide a highly sensitive, non-destructive means for identification of a compound, particularly when coupled with other information such as retention times or co-chromatography with reference compounds[1-4]. The technique of continuous-flow UV scanning has been shown to be ideally suited to identify and measure small, closely spaced chromatographic peaks. For maximum sensitivity and accurate interpretation of data, appropriate background corrections, whether manual or electronic, are required for each spectral scan.

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REFERENCES

1 A. M. Krstulovic, P. R. Brown and D. M. Rosie, *Anal. Chem.*, 48 (1977) 1383.
2 A. M. Krstulovic, P. R. Brown and D. M. Rosie, *Anal. Chem.*, 49 (1977) 2237.
3 A. M. Krstulovic, R. A. Hartwick, P. R. Brown and K. Lohse, *J. Chromatogr.*, 158 (1978) 365.
4 R. Yost, J. Stovenken and W. Maclean, *J. Chromatogr.*, 134 (1977) 73.
5 H.-J. Breter and R. K. Zahn, *J. Chromatogr.*, 137 (1977) 61.
6 J. K. Baker, R. E. Skelton and C.-Y. Ma, *J. Chromatogr.*, 168 (1979) 417.
7 A. M. Krstulovic, R. A. Hartwick and P. R. Brown, *Clin. Chem.*, 25 (1979) 235.
8 J. Readman, L. Brown and M. Rhead, *Analyst (London)*, 106 (1981) 122.
9 R. A. Hartwick, A. M. Krstulovic and P. R. Brown, *J. Chromatogr.*, 186 (1979) 659.
10 H. W. Shmukler, *J. Chromatogr. Sci.*, 8 (1970) 581.
11 H. R. Petty, B. R. Ware, L. F. Liebes, E. Pelle and R. Silber, *Blood*, 57 (1981) 250.
12 J. Donofrio, M. S. Coleman and J. J. Hutton, *J. Clin. Invest.*, 62 (1978) 884.
13 M. McKeag and P. R. Brown, *J. Chromatogr.*, 152 (1978) 253.
14 P. R. Brown, A. M. Krstulovic and R. A. Hartwick, *Advan. Chromatogr.*, 18 (1980) 101.
15 L. N. Klatt, *J. Chromatogr. Sci.*, 17 (1979) 225.