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Note

Dyes permit immediate evaluation of high-performance liquid chromatographic system performance

Application to high-performance liquid chromatography with radioimmunoassay detection

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Reversed-phase high-performance liquid chromatography (HPLC) is a powerful tool for separating peptides with similar amino acid sequences [1—3]; however, minor changes in either mobile phase composition or flow-rate may affect retention time [4]. When eluted peptides are quantitated by radioimmunoassay (RIA), several days are generally required before HPLC malfunctions become evident. Although it is possible to evaluate column performance with standards supplied by a column manufacturer or with a tryptic digest of a known protein, it is advantageous to evaluate performance with the conditions to be used for experimental samples. Furthermore, it is desirable to have an indicator permitting rapid verification that the HPLC system is functioning properly during each separation. The present investigation characterizes the retention times of several commercially available dyes and demonstrates that phenol red can be used as a preliminary indicator of HPLC performance during HPLC with RIA detection of α-melanocyte stimulating hormone (αMSH)-like peptides in biological samples.

MATERIALS AND METHODS

A Beckman (Berkeley, CA, U.S.A.) Model 334 high-performance liquid chromatograph consisting of two Beckman 110A pumps, a Beckman 421

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controller, an Hitachi 155 variable-wavelength spectrophotometer and a LKB 2111 Multirac fraction collector was used for these studies. For chromatography using an octadecylsilane (ODS) column (Beckman Ultrasphere ODS, 250 × 4.6 mm, 5 μm particle size), the aqueous component of the mobile phase was 0.2 *N* formic acid adjusted to pH 3.2 with triethylamine (TEAF) or 0.2 *N* phosphoric acid adjusted to pH 3.2 with triethylamine (TEAP) [5]. For chromatography using a cyano (CN) column (Waters μBondapak CN, 300 × 3.9 mm, 10 μm particle size) the TEAP buffer was diluted ten-fold before use [6]. Acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) was the organic solvent used for all chromatographic separations.

Dyes were obtained from the following sources: methyl green (Fisher Scientific, Fairlawn, NJ, U.S.A.), phenol red, bromphenol blue, acid red 150, sudan orange G, brilliant green, crystal violet and Coomassie brilliant blue G (Sigma, St. Louis, MO, U.S.A.) and May Grunwald (Allied Chemical, Morristownship, NJ, U.S.A.). All dyes except bromphenol blue and sudan orange G were prepared at a concentration of 0.1 mg/ml in 2 *N* acetic acid. Bromphenol blue and sudan orange G were dissolved in TEAF—acetonitrile (79:21). The dye solutions were centrifuged before being injected into the chromatograph. Phenol red appears to be of sufficiently high quality that additional purification before use is not necessary. Some of the other dyes may require additional purification (i.e. Waters Sep-Pak) before routine use.

Enzymatically dispersed rat intermediate lobe cells were resuspended (100,000 cells/ml) in 1 ml of 2 *N* acetic acid containing the protease inhibitors iodoacetamide and phenylmethylsulphonyl fluoride [5]; this cell suspension was sonicated and diluted 20-fold with 2 *N* acetic acid containing the protease inhibitors and phenol red (0.1 mg/ml). A 100-μl aliquot of this solution was injected into the HPLC system; the gradient profiles and flow-rates are specified in the captions to the figures and tables. The mobile phase in each fraction was evaporated to dryness in a vacuum centrifuge (Savant Instruments, Hicksville, NY, U.S.A.) and the amount of immunoreactive-αMSH (IR-αMSH) was determined as described previously [5]. The presence of phenol red in the eluent from the chromatograph was either monitored spectrophotometrically at 254 nm or visually by the yellow color in some of the HPLC fractions.

RESULTS

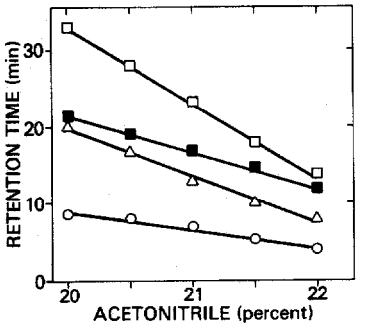
Several dyes eluted with different retention times from either an ODS or CN column (Table I). Using an ODS column, the retention times of the dyes were slightly longer with the TEAF buffer than with the TEAP buffer, however, the rank order of dye retention time remained the same. Using an ODS column with TEAF—acetonitrile as the mobile phase, the retention time of phenol red and the three forms of IR-αMSH were a linear function of acetonitrile concentration (Fig. 1). When the acetonitrile concentration was 21%, the three forms of IR-αMSH occurring in the dispersed intermediate lobe cells (which comigrated with synthetic desacetyl-αMSH, αMSH and N,O-diacetyl-αMSH) were resolved (Fig. 2); the phenol red did not interfere with the RIA (data not shown).

TABLE I

RETENTION TIMES OF DYES IN CHROMATOGRAPHIC SYSTEMS

A linear gradient consisting of the indicated aqueous component and acetonitrile (5% to 100%) was run over 95 min at a flow-rate of 1 ml/min and 1-ml fractions were collected. The data represent the retention time of each dye in the indicated chromatographic system.

Dye	Retention time (min)		
	ODS column		CN column
	TEAF	TEAP	TEAP
Methyl green	26	25	23
Phenol red	30	29	17
May Grunwald	37	32	24
Bromphenol blue	49	46	33
Coomassie brilliant blue G	52	49	40
Sudan orange G	54	50	33
Acid red 150	56	53	38
Crystal violet	67	64	44
Brilliant green	76	71	50



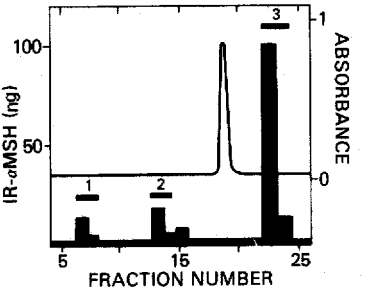


Fig. 1. Changes in retention time of the αMSH-like peptides and phenol red as a function of the acetonitrile concentration. The αMSH-like peptides from rat intermediate lobe cells and phenol red were resolved using an ODS column and a mobile phase consisting of an isocratic solution of acetonitrile and TEAF buffer (as indicated); the flow-rate was 1 ml/min. The presence of desacetyl-αMSH (○—○), αMSH (△—△) and N,O-diacetyl-αMSH (□—□) in the HPLC fractions were determined by RIA. The presence of phenol red (■—■) in the HPLC fractions was determined visually. For each acetonitrile concentration, the peak retention time of each compound is presented.

Fig. 2. Chromatographic resolution of the αMSH-like peptides and phenol red. The αMSH-like peptides from intermediate lobe cells and phenol red were resolved on an ODS column with the mobile phase TEAF—acetonitrile (79:21) at a flow-rate of 1 ml/min. The filled horizontal bars above the chromatographic fractions indicate the retention time of synthetic αMSH-like peptides.

DISCUSSION

Phenol red provides an example of a dye which can be used as an indicator of HPLC performance under specific chromatographic conditions. Routinely,

we determine the retention time of phenol red several times before injecting biological samples. After adequate performance of the HPLC system is verified, biological samples containing phenol red are injected. Using this protocol, HPLC system malfunctions can be determined rapidly. For example, check valve failure, decreased column efficiency or a partially obstructed frit result in phenol red retention time variation or the presence of phenol red in more than two 1-ml fractions. Once a problem is identified, procedures to improve system performance can be immediately initiated. Phenol red may not be the dye of choice for all chromatographic systems, however one or more of the dyes listed in Table I may be suitable as an indicator of HPLC performance in other chromatographic systems.

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