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Note

Guide to the selection of detecting agents for indirect photometric detection of aliphatic alcohols and other compounds by reversed-phase high-performance liquid chromatography

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It is possible to detect unionised non-UV absorbing solutes eluting from a reversed-phase high-performance liquid chromatographic (RP-HPLC) column by the addition of either an ionised[1–4] or an unionised[5–8] UV–VIS absorbing compound to the eluting solvent and using indirect photometric detection. The detection response is a complex function of both the chemical nature of the detecting agent and the eluting solute and the relative retention times of the two compounds, greatest sensitivity being achieved when the solute is eluted just prior to the retention time of the detecting agent[1,2,5,7,8]. As the detection response is poor, any co-elution of trace amounts of strongly UV-absorbing compounds may give rise to interference[6].

To render the technique generally applicable, it is necessary to develop a rational approach to both the selection of detecting agent and eluting solvent composition. For a compound to be useful routinely as a detecting agent, it must be selected such that it will elute prior to the solutes (affording negative peaks) or after the solutes (affording positive peaks)[1,2,4,5,7,8] and possess a retention time no greater than 10–15 min ($k' = 10$ approx.), so that baseline perturbation associated with the system peak[4–8] does not interfere with subsequent analyses. The potential for interference from trace amounts of strongly UV-absorbing co-eluants could be minimised by the provision of a number of detecting agents with similar chromatographic properties but widely differing spectral characteristics, so that a monitoring wavelength can be chosen where such interference should be minimised.

In this study four complementary homologous series of compounds have been synthesized and evaluated as detecting agents. These were 4-hydroxybenzoate (4-HB) esters (methyl to butyl); 4-aminobenzoate (4-AB) esters (methyl to dodecyl); 3,5-dinitrobenzoate esters (3,5-DNB) (methyl to dodecyl); and 2,4-dinitrophenylhydrazones (2,4-DNPH) of simple aliphatic aldehydes and ketones.

A procedure has been devised for the selection of both the composition of eluting solvent and detecting agent to enable a diverse range of aliphatic compounds to be examined in the indirect photometric mode.

EXPERIMENTAL

Reagents and materials

The alcohols, esters, ketones, 4-nitrobenzoyl chloride, 3,5-dinitrobenzoyl chlo-

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ride and 2,4-dinitrophenylhydrazine were obtained from Ajax Chemicals (Sydney, Australia) or BDH (Poole, U.K.). The 4-HB esters were obtained from Aldrich Chemical (Milwaukee, WI, U.S.A.). The 4-AB esters were prepared by reaction of 4-nitrobenzoyl chloride with excess alcohol followed by catalytic hydrogenation of the crude 4-nitrobenzoyl esters[9]. The 3,5-DNB esters and 2,4-DNPHs of the aldehydes and ketones were prepared by conventional techniques[10]. All compounds were recrystallised from either ethanol–water or ethyl acetate–hexane and shown to be chromatographically pure prior to use.

Chromatographic equipment

The liquid chromatograph consisted of a pump and variable-wavelength detector (LC-3, Pye-Unicam, Cambridge, U.K.), 20-μl loop injector (Rheodyne 7125, Cotati, CA, U.S.A.), integrating recorder (Hewlett-Packard 3380 A, Palo Alto, CA, U.S.A.) and a μBondapack C_{18} column (30 cm × 6.4 mm I.D.., 10 μm particle size) (Waters Assoc., Sydney, Australia).

Determination of partition coefficients

The partition coefficients between 1-octanol and water (P) for methyl 3,5-DNB and the 2,4-DNPHs of formaldehyde and acetone were determinated by shaking an 0.1% (w/v) aqueous solution (20 ml) with water-saturated 1-octanol (5 ml) until equilibrium was achieved, separating the layers by centrifugation at 1000 g and assaying by RP-HPLC both the aqueous layer and the 1-octanol layer following dilution with methanol. Methyl 3,5-DNB and formaldehyde 2,4-DNPH were analysed by using 50% methanol and monitoring at 228 and 360 nm respectively and acetone 2,4-DNPH by the use of 70% methanol monitoring at 367 nm. All determinations were in duplicate and results were consistent. Other values were either abstracted from the literature[11] or calculated from the above values by the use of established additivity rules[11].

The log P values for the homologous series of aliphatic alcohols 1-propanol to 1-dodecanol were derived by abstracting data from the literature[11]. The values for 1-propanol to 1-octanol were used (with the exception of the value for 1-butanol which appeared anomalous) to obtain a linear plot of log P *versus* carbon number [(log P) = 0.5358 (carbon No.) − 1.257 (r = 0.990)] and the values 1-propanol to 1-dodecanol calculated from this line.

Determination of capacity factors

Capacity factors (k') were determined by duplicate injections of compounds and calculated by the formula:

$$k' = \frac{t - t_0}{t_0}$$

where t is the retention time of the substance and t_0 the retention time of sodium nitrite, all determinations being made at a flow-rate of 1.5 ml min^{-1}. The values of t for 4-HB, 4-AB, 3,5-DNB esters and the 2,4-DNPHs were determined by injection of 20 μl of a $5 \cdot 10^{-4}$ M solution of the compounds in mobile phase and monitoring at their λ_{max}. The value of t for the aliphatic alcohols was determined by injection of

a 1% (w/v) solution of the alcohol in mobile phase and monitoring by indirect photometric detection using an appropriate detecting agent. The range of methanol compositions used as solvents were 50, 60, 70, 80 and 90%. The 4-HB esters were also measured using 40% and the alcohols using 75% methanol.

RESULTS AND DISCUSSION

The criteria used in the selection of the four classes of detecting agents used in this study were: (a) all readily synthesized in pure crystalline form from cheap and readily available starting materials; (b) having a broad range of polarities within each series so being eluted from RP-HPLC columns using a wide range of solvent methanol compositions, and giving peaks of good shape with no tailing; (c) having readily calculable log *P* values so that their elution characteristics can be predicted; (d) having spectral characteristics such that the four series compliment each other so that monitoring wavelengths over the range 220–400 nm can be used whilst the spectra within each series are relatively constant, making wavelength selection easier; (e) possessing high optical absorptivities so that they can be used at low concentration in the eluting solvent so as to minimise interference with the chromatographic characteristics of solutes under study.

For each series of detecting agents the plot of log *k'* *versus* log *P* produced an approximately linear relationship at all methanol compositions studied. Such relationships have been previously noted for homologous series of compounds on RP-HPLC[12]. However, results between different series were less consistent, producing a series of approximately parallel lines (Fig. 1 for results in 70% methanol which is representative). Inconsistency between log *P* and log *k'* on RP-HPLC for diverse compounds has been previously noted[13–19] and attempts to relate the two have met with only limited success. This inconsistency precluded the direct use of log *P* as a means of selecting a detecting agent most suitable for the detection of an aliphatic

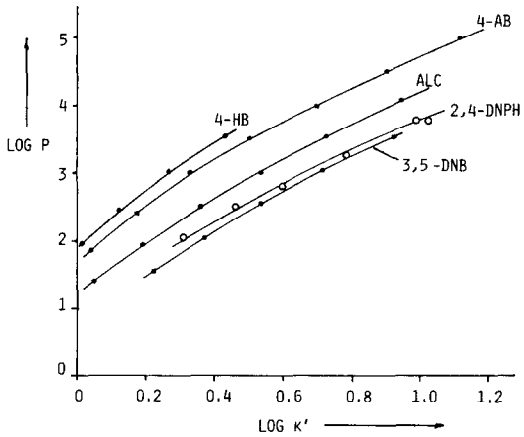

Fig. 1. Plot of log *P* *versus* log *k'*. 4-HB, 4-hydroxybenzoate esters (methyl to butyl); 4-AB, 4-aminobenzoate esters (ethyl to octyl); 3,5-DNB, 3,5-dinitrobenzoate esters (methyl to pentyl); 2,4-DNPH, 2,4-dinitrophenylhydrazones (formaldehyde, acetaldehyde acetone, methyl ethyl ketone, methyl propyl ketone and diethyl ketone); ALC, aliphatic alcophols (1-propanol to 1-octanol). Solvent 70% methanol.

solute. In general, the best that could be selected would be approximately ± 1 log P unit from the log P of the alcohol series (*i.e.* approximately two members above or below the desired compound assuming that each methylene group has a value of 0.5[11]) (Fig. 1). The line for the homologous series of aliphatic alcohols falls at the approximate mean for the four series of detecting agents for all solvent compositions. Therefore, the detecting agents were assigned a value relative to the log P of the alcohol series (termed log $P_{alc.}$) where

$$\log k' \text{ (detecting compound)} = \log k' \text{ (alcohol)}$$

by calculation from the graphs of log P *versus* log k' at different solvent compositions. These values were consistent over the complete range of solvent methanol compositions studied and differed by no more than ±0.15 units from the mean values. This was considered to be sufficiently accurate to enable these values to be used to select detecting agents. Fig. 2 shows these relationships and also the relationships between methanol composition and observed log k' for the alcohol series. By reading the figure horizontally information can be derived for a solute alcohol (the log P of which may be either calculated by additivity rules or abstracted from the literature[11]) and the detecting agents available which will elute either just prior to or just after the solute to be detected. The figure also serves as a rough guide to the solvent methanol composition to be used for the analysis.

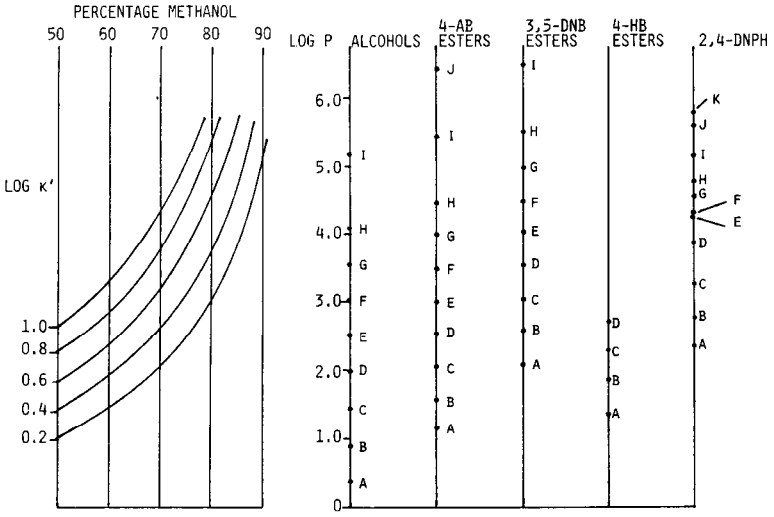

Fig. 2. Relationships between log k' percent methanol composition of mobile phase, log P of aliphatic alcohols and log P_{alc} for various detecting agents. Alcohols: A = 1-propanol; B = 1-butanol; C = 1-pentanol; D = 1-hexanol; E = 1-heptanol; F = 1-octanol; G = 1-nonanol; H = 1-decanol; I = 1-dodecanol. 4-Aminobenzoate (4-AB), 3,5-dinitrobenzoate (3,5-DNB) and 4-hydroxybenzoate (4-HB) esters: A = methyl; B = ethyl; C = propyl; D = butyl; E = pentyl; F = hexyl; G = heptyl; H = octyl; I = decyl; J = dodecyl. 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives: A = formaldehyde; B = acetaldehyde; C = acetone; D = methyl ethyl ketone; E = methyl propyl ketone; F = diethyl ketone; G = methyl isobutyl ketone; H = ethyl propyl ketone; I = methyl pentyl ketone; J = camphor; K = diisobutyl ketone.

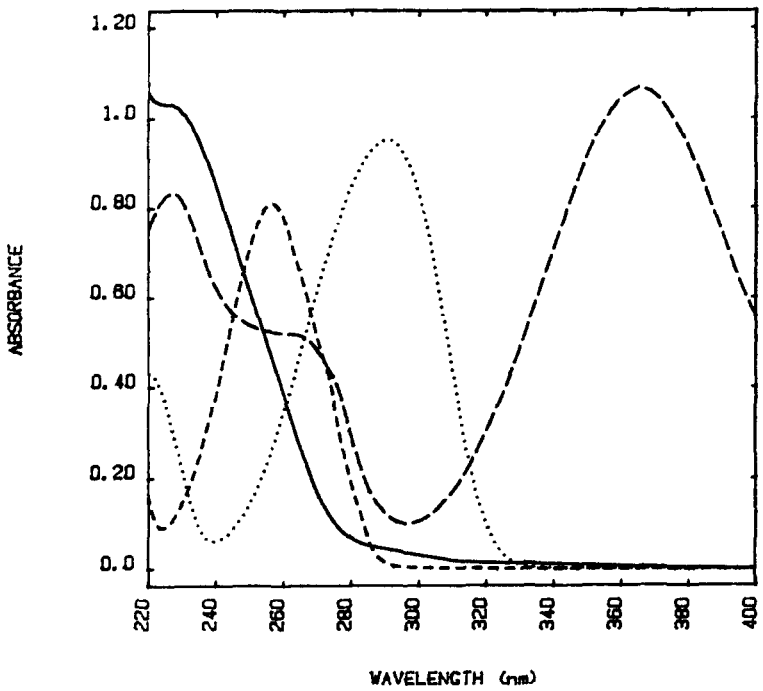

Fig. 3. Spectral characteristics of representative detecting agents. (·····), Ethyl 4-aminobenzoate; (----), methyl 4-hydroxybenzoate; (———), methyl 3,5-dinitrobenzoate; (– – –), 2,4-dinitrophenylhydrazone of acetone. Solvent: 70% ethanol; concentration: $5 \cdot 10^{-5}$ *M*.

The general utility of the detecting agents was evaluated using pentyl 3,5-DNB, heptyl 4-AB and the 2,4-DNPH of methyl propyl ketone in 75% methanol and propyl 4-AB, ethyl 4-HB, methyl 3,5-DNB and the 2,4-DNPH of formaldehyde in 50% methanol. All agents studied effectively detected aliphatic alcohols, esters, ethers and ketones, and it is reasonable to suppose that the other members of the four series would be equally effective in this role. The concentration of detecting agent used was generally in the range ($0.5 \cdot 10^{-5}$–$2 \cdot 10^{-5}$ *M*) which, when monitored at the wavelength of maximum absorbance of the detecting agent (Fig. 3), afforded a background absorbance to the eluting solvent which could be adequately compensated for by the instrumentation. Other wavelengths could be used with appropriate adjustment of concentration of detecting agent. The wide range of spectral characteristics enables a monitoring wavelength to be chosen at which interference from other UV-absorbing co-eluted solutes is minimised. The 2,4-DNPHs appear to be particularly useful agents as, when monitored in the region 360–370 nm, they suffer little interference. An example of this application is the detection of menthol in Menthol and Pine Inhalation APF[20] [menthol 2% (w/v), pumilio pine oil 5% (v/v) in 90% ethanol]. A previously reported HPLC assay for menthol in Menthol Inhalation APF[20] [2% (w/v) menthol in 90% ethanol] using heptyl 4-AB and monitoring at 290 nm[6] is not adaptable to the more complex formulation as considerable interference occurs from strongly UV-absorbing components of the pumilio pine oil (Fig. 4a). This interference is absent when the 2,4-DNPH of methyl propyl ketone is used as detecting agent

(Fig. 4b) and, as these co-eluting compounds from the oil are present in only small amounts, they are not detected by the agent.

The use of log P to select solvent composition and detecting agent for aliphatic compounds other than alcohols is demonstrated by the data presented in Table I. It appears that aliphatic compounds in general elute at retention times calculable from log P so that Fig. 2 can be used as a general predictor of desirable solvent composition and detecting agent for a broad range of aliphatic compounds.

In all of the systems studied the retention time of the solutes was unaffected by the nature of the detecting agent. This confirms previous data from this laboratory which showed, with low-molecular-weight alcohols, that retention time of the solutes was unaffected by both the nature and concentration of the detecting agent[7]. This

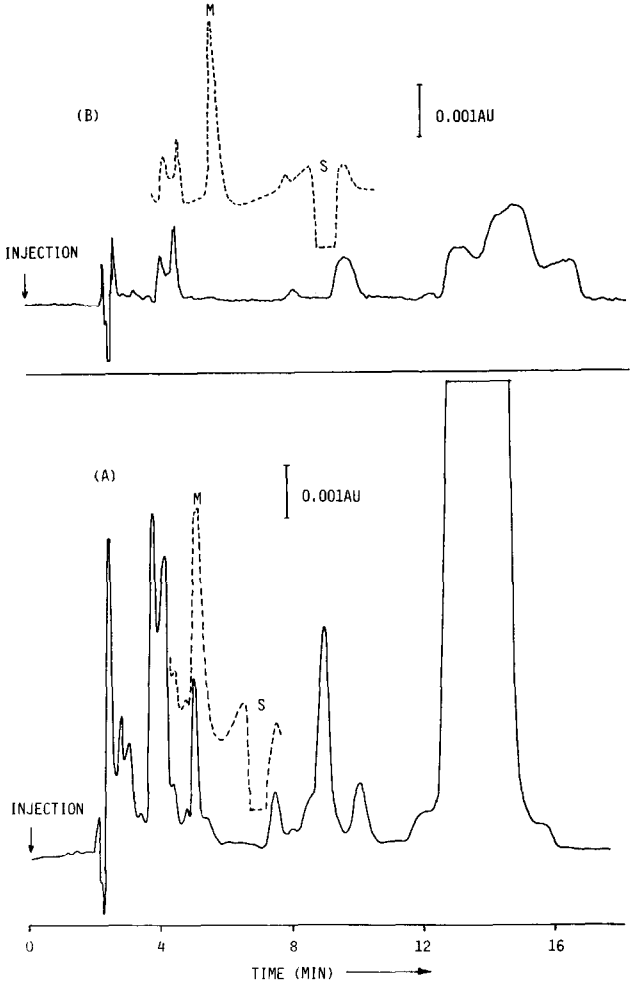

Fig. 4. Chromatograms of Menthol and Pine Inhalation A.P.F. (20 μl) in the presence (– – –) and absence (———) of detecting agent. Solvent: 75% methanol. Flow-rate: 1.5 ml min^{-1}. (A) Detecting agent: heptyl 4-AB ($5 \cdot 10^{-6}$ M) monitored at 290 nm and (B) detecting agent: 2,4-DNPH of methyl propyl ketone ($5 \cdot 10^{-6}$ M) monitored at 367 nm. M = Menthol; S = system peak.

TABLE I

RESULTS OBTAINED BY THE USE OF FIG. 2 TO PREDICT RETENTION TIME, SOLVENT COMPOSITION AND OPTIMUM DETECTING AGENT FOR A REPRESENTATIVE RANGE OF ALIPHATIC COMPOUNDS

Compound	*Log P*	*Observed retention time (min)*	*Calc. retention time (min)*	*Solvent composition (percentage methanol)*	*Detecting agent $1 \cdot 10^{-5}$ M*	*Retention time of detecting agent (min)*	*Monitoring wavelength (nm)*
Menthol	3.3*	4.73	4.8	75	Heptyl 4-AB	6.19	290
Ethyl heptanoate	3.2**	8.82	6.5	70	Methyl propyl ketone 2,4-DNPH	13.70	367
Amyl acetate	2.2**	4.64	3.7	70	Acetone 2,4-DNPH	7.00	367
Methyl amyl ketone	1.8**	3.74	4.0	70	Acetone 2,4-DNPH	7.00	367
Diisobutyl ketone	2.4**	5.82	4.3	70	Acetone 2,4-DNPH	7.00	367
Butyl acetate	1.7**	7.28	6.1	50	Propyl 4-AB	7.78	290
Ethyl acetate	0.7*	3.22	3.1	50	Ethyl 4-HB	6.75	256

* Literature values (ref. 11).
** Calculated.

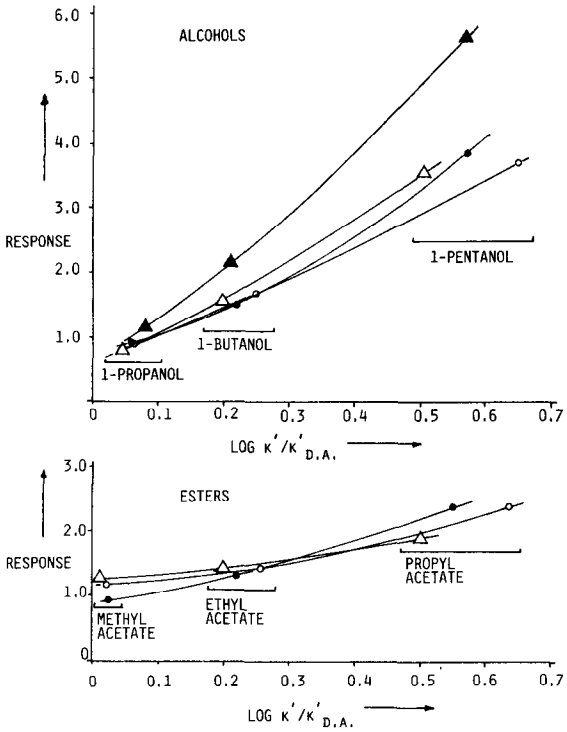

Fig. 5. Plot of relative response (area response/molar extinction coefficient at detecting wavelength) *versus* log $k'_{(\text{solute})}/k'_{(\text{detecting agent})}$ for aliphatic alcohols and esters. Solvent: 50% methanol containing $5 \cdot 10^{-6}$ *M* detecting agent. ▲—▲, methyl 3,5-DNB monitored at 228 nm; △—△, formaldehyde 2,4-DNPH monitored at 360 nm; ●—●, propyl 4-AB monitored at 290 nm and ○—○, ethyl 4-HB monitoring at 260 nm. Injection: 20 µl of (1%, w/v) solution. D.A. = Detecting agent.

suggests that when these agents are used at low concentration ($0.5 \cdot 10^{-5}$–$2 \cdot 10^{-5}$ *M*) they leave the chromatographic characteristics of the RP-HPLC column unchanged and that retention data using agents from different series can be directly compared. As has been observed previously, the response for all compounds is a complex function of the nature of the detecting agent and eluted solute and sensitivity increases as the retention time of the test solute approaches that of the detecting agent[1,4,5,7,8]. Fig. 5 illustrates the results obtained for four detecting agents in 50% methanol as eluting solvent. By dividing the area response by the molar extinction at the monitoring wavelength, a comparative assessment can be made of response *versus* log k' relative to the log k' of the detecting agent. No attempt has been made in these studies to validate the linearity of response to varying concentrations of solutes. However, previous studies have shown that indirect photometric detection can be used quantitatively[6,7]. The techniques proposed here provide a means of rationally selecting detecting agents which enable the variable-wavelength UV-detector to be used for the non-specific general purpose detection, and presumably quantitation, of aliphatic non-UV absorbing compounds such as alcohols and UV-absorbing compounds such as ketones using wavelengths where they fail to absorb naturally.

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