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Note

Simple method for the optimisation of mobile phase composition for high-performance liquid chromatographic analysis of a multi-component mixture

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A major problem often encountered by the analyst, is the development of a high-performance liquid chromatographic (HPLC) assay of a multi-component mixture, with optimal separation in a minimal elution time. After selection of the column conditions, reversed- or normal-phase, and a possible eluent, the choice of the mobile phase composition appears frequently to be one of trial and error. Several attempts have been made at providing a systematic approach toward the optimisation of solute resolution in HPLC. Some authors have addressed themselves to this by considering the effects of mobile phase composition on solute retention[1,2], whilst others have used computers to facilitate optimisation with a regard to factors in addition to mobile phase composition such as temperature and column efficiency[3,4]. All these methods require either a considerable volume of data or computation.

The present paper proposes a simple graphical method for the rapid selection of an optimal mobile phase composition of a binary mixture. The approach is illustrated by the separation of a multi-component mixture of barbiturates using reversed-phase HPLC and an acetonitrile–water mixture as the mobile phase.

EXPERIMENTAL

Six barbituric acid derivatives were obtained from commercial sources or were synthesised from malonic ester, via the diethyl-2-alkyl-2-ethylmalonate intermediate. The structures and melting points of the barbiturates are listed in Table I.

The HPLC chromatographic system used was based on that described by Clark and Chan[5]. Separation of the non-ionised barbituric acids was carried out on a 30 × 0.5 cm, C_{18} reversed-phase column (Hypersil ODS/5 μm), with solvent being delivered at a flow-rate of 1 ml/min by a Waters M6000 dual reciprocating pump. Conversion of the non-ionised to the more strongly absorbing monoanionic species was achieved by post-column introduction of pH 10 borate buffer, delivered at flow-rate of 0.2 ml/min via a Parker Hannifin "Tee piece" (1/16 in.) from a second Waters M6000 dual reciprocating pump.

Detection of the ionised barbiturates was achieved using a Waters 440 absorbance detector set at 254 nm. All injections were made using a Waters U6K loop injector and chromatograms were obtained using a Servoscribe 210 recorder.

The mobile phase was an acetonitrile–water mixture. Acetonitrile was of HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain) and the various sol-

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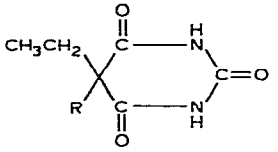
TABLE I
BARBITURIC ACID DERIVATIVES AND THEIR RESPECTIVE MELTING POINTS



Key	*Compound*	*R*	*M p (°C)**
1	5-Ethyl-5-*n*-propylbarbituric acid	$CH_3(CH_2)_2$–	144–146
2	5-Ethyl-5-(1′-methylbutyl) barbituric acid (pentobarbitone)	$CH_3(CH_2)_2CH(CH_3)$–	127–129
3	5-Ethyl-5-*n*-hexylbarbituric acid	$CH_3(CH_2)_5$–	124–126
4	5-Ethyl-5-*n*-heptylbarbituric acid	$CH_3(CH_2)_6$–	117–119
5	5-Ethyl-5-*n*-octylbarbituric acid	$CH_3(CH_2)_7$–	112–114
6	5-Ethyl-5-*n*-nonylbarbituric acid	$CH_3(CH_2)_8$–	104–106

* Melting points were determined using a Kofler hot-stage apparatus.

vent systems were prepared for use by sonication and/or filtration using a 0.22-μm Millipore filter.

RESULTS AND DISCUSSION

The problem was to find as rapidly as possible the acetonitrile–water composition that gives optimum resolution of the six barbiturates listed in Table I. The solution lay in the empirical observation that for all barbiturates studied, a linear relationship existed between log $(1 - R_0/R_T)$, here designated R_Q, and the mobile phase composition (Fig. 1), where R_0 is the time for elution of the unretained solute

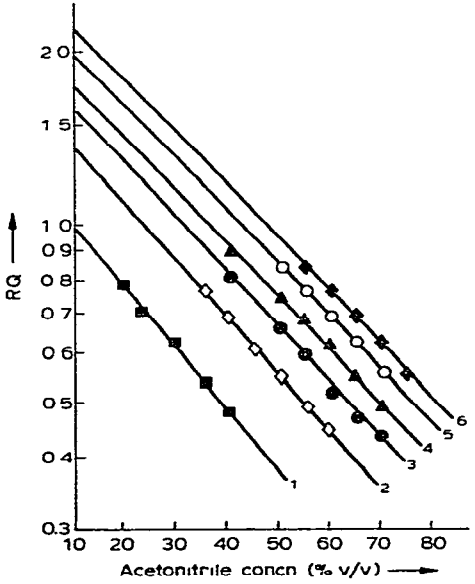
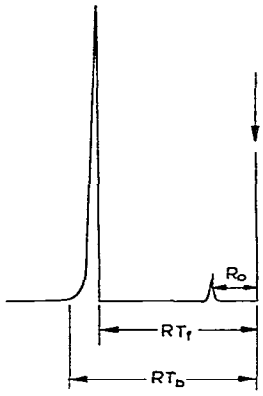


Fig. 1. Plot of R_Q values of barbituric acid derivatives against the concentration of acetonitrile in the mobile phase. See Table I for key.

peak (solvent front) and R_T is the time for elution of the solute. As expected, with the homologous barbiturate series studied, for a given acetonitrile–water composition, the higher the molecular weight and lipophilicity, the longer the retention time on the C_{18} column.

The application of the linear relationship noted in Fig. 1 to optimal mobile phase selection can perhaps be best understood by considering the separation of a two-component mixture, pentobarbitone and 5-ethyl-5-*n*-hexylbarbituric acid. Optimum resolution means complete separation of the solute peaks at their base in the shortest possible time. A practical approach to achieving this goal is to plot R_Q values, estimated for both the front, $R_{Q\text{front}}$, and the back, $R_{Q\text{back}}$, of the solute peaks, against percentage solvent composition (Fig. 2), and to define optimum resolution as the point where the plots of $R_{Q\text{front}}$ for one solute intersects with that of the $R_{Q\text{back}}$ for the other. This approach can be applied even when the peaks are asymmetric. As seen in Fig. 3, optimum resolution of pentobarbitone and 5-ethyl-5-*n*-hexylbarbituric acid is achieved with acetonitrile–water (1:1) (Fig. 4).



$RQ(\text{front}) = \log(1-R_o/RT_f)$

$RQ(\text{back}) = \log(1-R_o/RT_b)$

Fig 2 Derivations of $R_{Q\text{front}}$, $R_{Q\text{back}}$ from a chromatographic trace

Extension of this approach to the six component barbiturate mixture is relatively simple. Examination of the plot of $R_{Q\text{back}}$ and $R_{Q\text{front}}$ *versus* percentage acetonitrile composition for the multicomponent system reveals four points of intersection (Fig. 5), that is four potential points of optimum separation. One of these points, that for pentobarbitone and 5-ethyl-5-*n*-hexylbarbituric acid, has already been discussed. Optimum separation may be achieved for 5-ethyl-5-*n*-nonylbarbituric acid and 5-ethyl-5-*n*-octylbarbituric acid, 5-ethyl-*n*-octylbarbituric acid and 5-ethyl-5-*n*-heptylbarbituric acid, 5-ethyl-5-*n*-hexylbarbituric acid and pentobarbitone at 70, 60 and 50% acetonitrile respectively. A point of interest and a deciding factor in the final choice of a suitable mobile phase is the point of intersection for pentobarbitone and 5-ethyl-5-*n*-heptylbarbituric acid. At 65% acetonitrile optimum resolution is achieved for pentobarbitone and 5-ethyl-5-*n*-heptylbarbituric acid, however, the point of intersection falls directly between the $R_{Q\text{front}}$ and the $R_{Q\text{back}}$ plots of 5-ethyl-5-*n*-

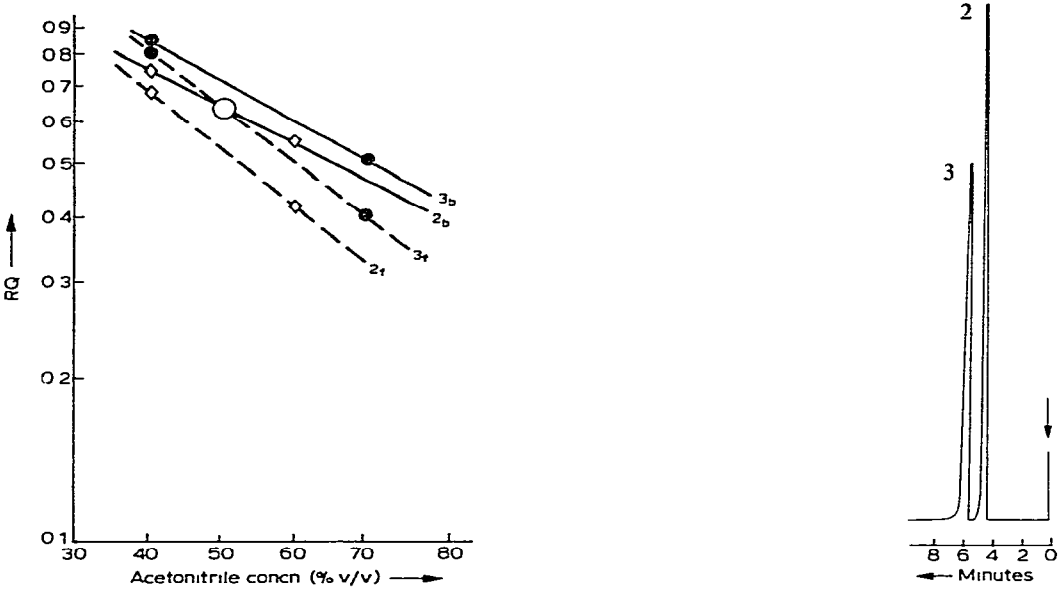


Fig 3 Plot of R_Q values, for front (f), and back (b), of pentobarbitone and 5-ethyl-5-*n*-hexylbarbituric acid against concentration of acetonitrile in the mobile phase See Table I for key

Fig 4 Chromatogram of pentobarbitone and 5-ethyl-5-*n*-hexylbarbituric acid showing optimum resolution at 50% acetonitrile in the mobile phase See Table I for key

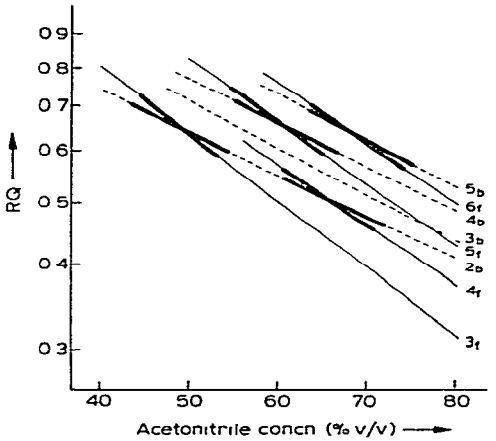


Fig 5. Plot of R_Q values, for front (f) (——), and back (b) (––), of the multi-component barbiturate mixture against concentration of acetonitrile in the mobile phase. The bold lines emphasize points of intersection See Table I for key.

hexylbarbituric acid indicating an unfavourable overlap of the peaks of the three compounds (Fig. 6). A suitable mobile-phase composition must therefore be below 65% acetonitrile and preferably between the three remaining points of optimum resolution. Selection of a mobile phase composition of 55% acetonitrile seems reasonable, and indeed gave a suitable separation of all six compounds with the elution pattern as predicted (Fig. 7).

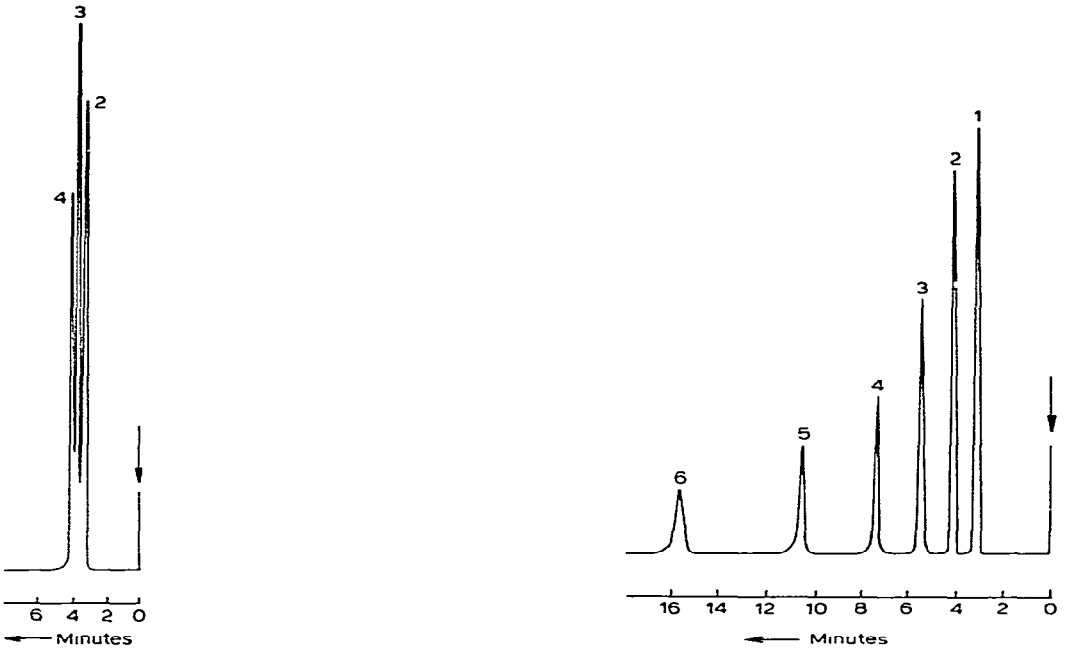


Fig. 6. Chromatogram of pentobarbitone, 5-ethyl-5-*n*-hexylbarbituric acid and 5-ethyl-5-*n*-heptylbarbituric acid at 65% acetonitrile in the mobile phase showing the unfavourable overlap between the three peaks See Table I for key

Fig. 7. Chromatogram showing the optimum resolution of the multi-component barbiturate mixture with a mobile phase composition of 55% acetonitrile in water. See Table I for key.

GENERAL DISCUSSION

In HPLC, a common method of assessing the performance of the system is to calculate the value of the capacity factor k' [$= (R_T - R_0)/R_0$]. Plots of log k' *versus* solvent composition are rarely linear[6]. Using the data from a recently reported example of thiamphenicol[7], employing a methanol–water mobile phase, a curvilinear relationship exists between log k' and either solvent composition (Fig. 8) or log solvent composition. In contrast, plots of R_Q were linear in all the cases we have examined including the thiamphenicol data. The utility of a linear function over a curvilinear function, when trying to interpolate intermediary values, is self-evident. The relationship between R_Q and k' is clearly $R_Q = \log k'/(1 + k')$ hence R_Q values can be readily calculated from k' values, and *vice versa*.

The method proposed for optimising mobile phase composition is a practical one employing simple and easily measured parameters. As the relationship between R_Q and solvent composition is linear only two points are necessary to construct the graph, that is to say, only one solvent change is required to gain information regarding the entire chromatographic system. This method of optimisation is very rapid as compared to the time involved in the many solvent changes required when employing more conventional methods for solvent selection. Any specification for optimum resolution is clearly a function of the relative concentrations of the materials in the

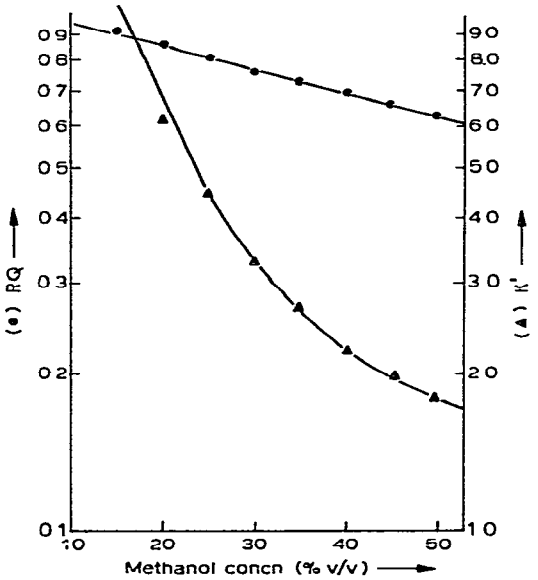


Fig. 8. Plots of R_Q and log k' for thiamphenicol against the concentration of methanol in the mobile phase, using the data of Crechiolo and Hill[7].

final mixture. In practice, one would examine the system using the most extreme concentrations anticipated of each component. If, during application, even more extreme concentrations are encountered, fine tuning of the system may be necessary.

Some compounds may not be resolved using a simple two-component mobile phase. Such instances may, however, be rapidly identified using the method proposed without unnecessary experimentation with the solvent system. Likewise, a lack of convergence of the values of $R_{Q\,\mathrm{back}}$ of one compound and $R_{Q\,\mathrm{front}}$ of another, rapidly indicates that there is no point in pursuing further various compositions of that particular binary mobile phase. Even if an optimum separation cannot be seen with a two-component mobile phase, an indication of the required solvent system with regard to polarity, etc. might be indicated from a study of the plot.

The method proposed is only applicable to a given HPLC system and does not take into account, unlike other methods[3], such factors as column efficiency and mobile-phase flow-rate. We believe, however, that this approach to the optimisation of solvent selection could be of use in the separation of multi-component mixtures other than that described and thus could reduce the unnecessary waste of solvents, time and effort associated with more conventional methods of mobile phase selection.

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