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Short Communication

Approaches to method development for trace organic analysis*

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Introduction

The determination of trace amounts of organic compounds has proved of immense value in many fields of research, including clinical chemistry [1], in ensuring safer and more effective drug therapy [2], in monitoring occupational and environmental exposure to chemicals [3] and in analytical toxicology [4]. Although few novel techniques are emerging, major advances in methodology have been achieved by adaptions and optimisation of existing techniques. In the biomedical field the successful development of a HPLC method often involves two distinct steps, optimisation of chromatographic conditions followed by selection of a suitable sample preparation procedure. For many compounds the development of HPLC conditions has proved to be relatively simple. A reversed-phase column (usually C-18 or C-8 bonded silica) has been used and the choice of mobile phase involved optimisation of the aqueous/modifier ratio, the pH, and the use of additives such as ion-pair reagents. The development of suitable sample preparation procedures has often proved the more challenging part of method development, particularly when dealing with biological matrices. The complexity of sample preparation depends on several factors such as the chemical nature of the compound(s) to be assayed, the sensitivity required, the specificity of the end step and the sample matrix. The most common sample preparation procedures are liquid–liquid extraction and solid-phase extraction which has been carried out both on-line and off-line [5]. With multistage sample preparation procedures, there are many pitfalls: losses or incomplete release from proteins can occur when precipitating plasma proteins; labile compounds such as drug metabolites may revert to the parent compound during sample work-up; emulsions can form when extracting with organic solvents; losses can occur when drying down solvents; contamination from glassware or reagents can occur; and impurities from

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reagents may interfere or even react with analytes. This brief review aims to highlight particular points of methodology that are crucial to the successful development of an analytical method. In all cases the methods have been applied to real samples.

Results

The effect of solvent composition on retention of propanolol is shown in Fig. 1 using both a capped (Hypersil ODS) and an uncapped (Sperisorb ODS) reversed-phase column. The methanol/water ratio was varied over the range 40–100% methanol in the mobile phase which also contained 0.1% v/v trifluoroacetic acid. As expected the retention decreased with increasing methanol concentrations, presumably due to the influence of the residual silanol groups on the uncapped phase.

When developing a method for the determination of butylated hydroxytoluene (BHT) in rat liver and tissue homogenates, the development of HPLC conditions was straightforward. The compound and its acidic, hydroxylated and aldehydic metabolites were all resolved and gave good peak shape on a 12.5 cm Spherisorb ODS −5 column (using 80% methanol in water as eluent). BHT is fairly liphophilic and thus extracted easily into organic solvents. However, experiments on spiked liver and tissue homogenates, extracted with various solvents and evaporated to dryness under nitrogen at room temperature, gave <10% recovery. On the other hand, evaporation to dryness under vacuum gave 90% recovery using ether as extractant. A specimen chromatogram of a tissue homogenate is shown in Fig. 2. The method was capable of detecting BHT down to at least 1 $\mu g\ ml^{-1}$ of homogenate.

When attempting to set up a method to determine the neuroleptic drug chlorpromazine (Fig. 3) in plasma some difficulty was encountered in achieving satisfactory chromatography even for standard solutions. On reversed-phase columns chlorpromazine (which is basic and fairly lipophilic) was too well retained even with high concentrations of organic modifier in the mobile phase. The use of a silica column with a typical reversed-phase type mobile phase (0.1% trifluoracetic acid in 70% methanol 30% water plus 10 mM sodium heptane sulphonate) gave good peak shape, short retention times and was reproducible over at least six months. In contrast, silica columns

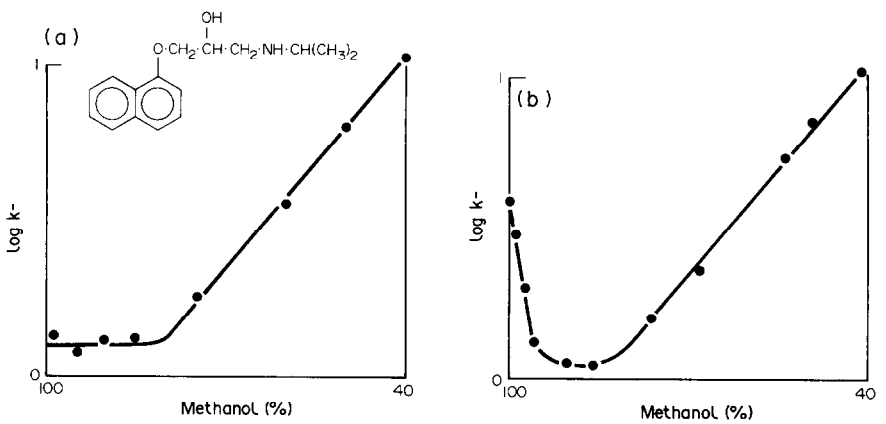

Figure 1
The effect of solvent composition on propranolol retention on (a) capped silica (Hypersil ODS) and (b) uncapped silica (Spherisorb ODS). All solvents contained 0.1% v/v trifluoracetic acid.

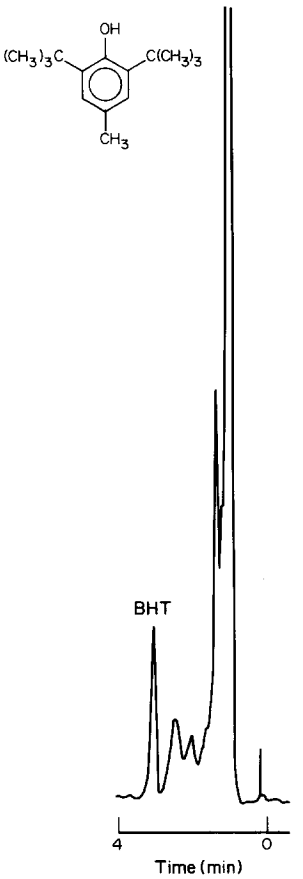

Figure 2
Specimen chromatogram of BHT extracted from tissue homogenate. Column: Spherisorb-5-ODS, 12.5 cm × 5 mm, eluent 80% methanol at a flow rate of 1.2 ml min^{-1}, UV detection at 279 nm. BHT concentration approx. 10 μg ml^{-1}.

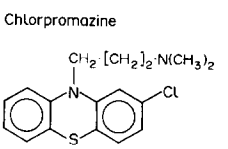

Figure 3
Structure of chlorpromazine.

tried in the conventional mode with low-polarity eluents gave irreproducible retention times. A full description of the method is given elsewhere [6]. Increasing the concentration of the pairing-ion decreased the retention of chloromazine and four of its metabolites.

The anti-estrogen tamoxifen has also been studied in these laboratories. It is also a lipophilic basic drug and gave poor peak shape on ODS, cyano and silica columns. The compound was well retained on reversed-phase columns and the addition of pairing-ions to the eluent would normally be expected to give increased retentions. However, surprisingly it was found that the addition of sodium heptane sulphonate to the eluent decreased retention and improved the peak shape of tamoxifen and its N-desmethyl metabolite on C-18, C-5 and C-8 silica columns. The effect on the retentions of tamoxifen and desmethyltamoxifen of adding heptane sulphonate to the mobile phase

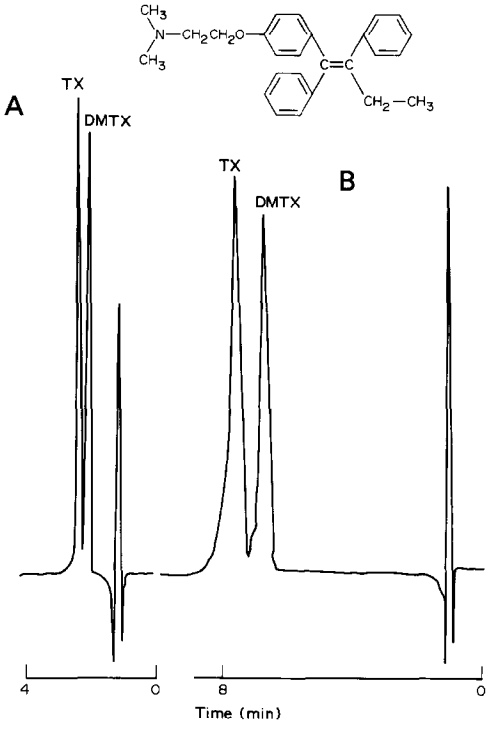

Figure 4
Chromatograms of tamoxifen (structure shown) and desmethyltamoxifen on C-5 silica (10 cm × 5 mm). Eluent 80% acetonitrile 20% pH 3 phosphate buffer (0.1 M) at 1.5 ml min^{-1}. UV detection at 240 nm. A with 10 mM sodium heptane sulphonate, B with no sodium heptane sulphonate in eluent.

with C-5 silica is shown in Fig. 4. Full details of the method have been published previously [7].

Discussion

Many facile HPLC separations can be set up by making logical changes to the composition of the mobile phase. It should always be remembered that solute retention may be via more than one mechanism as is evident by the influence shown by residual silanols (Fig. 1).

Due to its enormous versatility HPLC is very widely used for trace analysis and many elegant separations can and will continue to be demonstrated. Good separations can be achieved even when conventional separation mechanisms are not operating. For example, with tamoxifen the use of the pairing-ion to speed up and improve chromatography was the opposite to anticipated behaviour but was useful in practical terms. A possible explanation is that tamoxifen is a very lipophilic compound and the mobile phase was very rich (80%) in organic modifier. Ion-pairs formed in the mobile phase may have been competing with the sodium heptane sulphonate for sites on the stationary phase. In the case of chlorpromazine the successful use of a silica column with an aqueous mobile phase suggests that analysts should consider these systems for a wider range of compounds.

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