HPLC in clinical microbiology laboratories

High-performance (or pressure) liquid chromatography (HPLC) is an advanced form of liquid chromatography. In liquid chromatography a liquid solvent (the mobile phase) carries the mixture to be separated over an immobilized stationary phase upon which the chromatographic separation takes place. The mobile phase in HPLC is pumped rapidly and at high pressure through what is usually a highly efficient, microparticulate (5–10 \( \mu \)m) stationary phase held in a relatively narrow bore column. After separation the individual compounds are detected, often by ultraviolet absorption, characterized by the time they take to elute from the system (retention time) and quantitated by the measurement of the area or height of peaks from a chart recorder trace.

The major advantages of HPLC over conventional chromatography are better resolution, shorter analysis time and superior precision, reproducibility and sensitivity. A form of HPLC known as reverse phase chromatography utilizes a non-polar stationary phase (usually octadecylsilane functional groups chemically bonded to silica and referred to as O.D.S. or C\(_{18}\) packings) and a polar (watery) mobile phase. This is particularly useful for the analysis of body fluids since aqueous samples can be introduced directly into the chromatograph, and an enormous range of compounds of varying molecular weights and polarities can be separated on a single column by suitably modifying the mobile phase composition. In recent years there has been considerable interest in the measurement of antibiotics in body fluids by HPLC and the subject has been reviewed (Anhalt, 1978; Yoshikawa et al., 1980; White & Reeves, 1981). Therapeutic monitoring of aminoglycosides, chloramphenicol, and antifungals is the application for which a clinical laboratory is likely to consider HPLC.

Reverse phase methods have been described for assaying amikacin (Anhalt & Brown, 1978; Maitra et al., 1977), gentamicin (Anhalt, 1977; Maitra et al., 1977; Chiou et al., 1978), netilmicin (Peng et al., 1977b), and tobramycin (Anhalt & Brown 1977, Maitra et al., 1979; Haughey et al., 1980) in serum. All methods are relatively fast and specific and show good accuracy, precision and correlation with other methods but require large amounts of sample. Detection at the level of sensitivity required for clinical assays has required preparing fluorescent derivatives using either dansyl chloride (Peng et al., 1977a, b; Chiou et al., 1978) or o-phthalaldehyde, the groups using the latter reagent before (Maitra et al., 1977, 1978) or after (Anhalt & Brown, 1977) the HPLC separation. Pre-column derivation may theoretically give rise to multiple derivatives but this appears only to have occurred extensively with amikacin (Maitra et al., 1978). Post-column derivation eliminated this problem but required specialized equipment for introducing the derivative reagent at pressure into the mobile phase. HPLC of gentamicin is complicated by the fact that it is a mixture of four compounds (C\(_1\), C\(_{12}\), C\(_{13}\), C\(_{24}\)). Some methods separate C\(_1\), C\(_{12}\) and C\(_{13}\) and in others C\(_{12}\) and C\(_{13}\) co-elute. Component separation is not necessary for therapeutic monitoring and could cause inaccurate measurements if the calibrator solutions differed markedly in component ratio from the gentamicin in the samples. In this context no method for serum assays appears to resolve C\(_{24}\), although normally considered a minor component recent work shows that some brands of gentamicin may comprise up to 18% C\(_{24}\) (White & Reeves, manuscript in preparation). To sum up, HPLC, for therapeutic monitoring of amino-glycosides, seems to offer no particular advantages over other available methods and has the distinct disadvantage of requiring large samples of serum making it unsuitable for paediatric monitoring. Only laboratories where suitable equipment (with a fluorescence detector) and expertise are already available should seriously consider HPLC for monitoring aminoglycoside therapy. HPLC may of
course have application on the study of aminoglycoside pharmacology.

Chloramphenicol, unlike aminoglycosides, absorbs ultraviolet light and can be detected at therapeutic concentrations by a relatively inexpensive HPLC detector measuring absorbance at 254 or 280 nm. HPLC assay of chloramphenicol is very simple and quick, the only necessary sample preparation being the precipitation of serum proteins, although some authors prefer a solvent extraction step. Several reverse phase methods have been described (Nilsson-Ehle, Kahlmeter & Nilsson-Ehle, 1978; Koup et al., 1979; Sample et al., 1979, White & Reeves 1981). Parenteral chloramphenicol is administered as chloramphenicol succinate which is not itself microbiologically active and is rapidly hydrolyzed in vivo to the active chloramphenicol free base. HPLC will separate free base and succinate allowing quantitation of both; there are occasions where both forms may be present in serum (Nilsson-Ehle et al., 1978; White & Reeves, 1981). Solvent extraction as favoured by some authors (Koup et al., 1978; Sample et al., 1979) may improve assay specificity but if a patient's drug history is known and no known interfering drugs are present it seems unnecessary. A check for possible interference from other drugs is by monitoring at two wavelengths (254 and 280 nm). Interference manifests itself as a difference in the ratio: peak (280 nm)/peak (254 nm) of the calibrators to that of the tests.

In the field of antifungal drugs reverse phase HPLC assays have been described for amphotericin B (Nilsson-Ehle et al., 1977) flucytosine (Bury et al., 1979; Warnock & Turner, 1981) imidazoles (Brodie et al., 1978) and griseofulvin (Nation et al., 1978; Hackett & Dusci, 1978) and appear simple and suitable for therapeutic monitoring.

Thus reverse phase HPLC assays using C18 (O.D.S.) columns have been described for aminoglycosides, chloramphenicol and antifungals. Aminoglycoside assays apart they are rapid, straightforward, require only a basic instrument equipped with a suitable C18 column and an ultraviolet absorption detector, and are suitable for routine therapeutic monitoring. In addition for laboratories interested in research into antimicrobial agents and chemotherapy HPLC provides the means for specialist study of an enormous range of antibiotics and their metabolites (Yoshikawa et al., 1980; Reeves et al., 1980; White & Reeves, 1981). Finally it should be noted that the potential of HPLC for assisting in the diagnosis of infection is virtually unexplored.

L. O. WHITE
Department of Microbiology.
Southmead Hospital.
Bristol BS10 5NB, England

References
Bactericidal glycopeptide in human amniotic fluid

Human amniotic fluid (HAF) has been used as a negative control in standard assays of antibiotic activity (personal communication, Beecham Laboratories). It is ironic, therefore, that recent studies should have revealed that a low molecular weight glycopeptide found in HAF has potent antibacterial activity (Schleivert, Johnson & Galask, 1977; Sachs & Stern 1979). Its existence is, however, less than surprising because, although HAF has effective concentrations of lysozyme (Larsen, Galask & Snyder, 1973) and low concentrations of immunoglobulin and complement (Galask & Snyder, 1973), the relative freedom of HAF from bacterial infection has not been convincingly explained. Furthermore, the death of a fetus in utero is not inevitably followed by infection, even when the amniotic membranes are in contact with the vaginal space.

It has been noted that, in developing countries, there was a significantly higher incidence of amniotic infection, which was felt to be directly related to maternal malnutrition (Naeye & Blanc, 1970). In particular, some studies have suggested that a deficiency of zinc might be important. Early observations on the antibacterial effect of whole HAF were carried out on samples from Ethiopian women and demonstrated an increase in the proportion of non-inhibitory specimens, compared with HAF from American women (Naeye et al., 1977; Naeye 1977).

There followed an important series of papers from Schleivert and his colleagues (Galask, Schleivert & Johnson, 1975; Schleivert, Johnson & Galask, 1976). They isolated material of molecular weight between 650–750 daltons, protein in nature and whose antibacterial activity depended upon the presence of zinc (at a minimum of 0·1 mg/l), but was inhibitable by phosphate. This last fact explained its inactivity in antibiotic assays, where phosphate-containing media were used. Scanning electron microscopy of bacterial treated with this material suggested an effect upon bacterial cell wall growth. Their description of aminoacid analyses of purified material were slightly inconsistent as, although ultraviolet absorption at 280 nm was used to identify peaks of material on chromatography, no aromatic aminoacids were found.

These studies have been repeated and enlarged by Sachs and his collaborators (Sachs & Stern 1979; Sachs, Stern & Carroway, 1981), with some differences. They isolated material of a similar molecular weight, but with several other small contaminants which had no bactericidal activity. It was zinc dependent, phosphate inhabitable, but failed to absorb at 280 nm. Unlike Schleivert, they were unable to purify it by ion-exchange chromatography, but could detect it in HAF after 20 weeks gestation.

There was a wide range of bactericidal activity, both Gram-positive and Gram-negative bacteria being susceptible...
Leading articles

(Schleivert, Johnson & Galask, 1977; Sachs & Stern 1979; Larsen & Galask, 1975), at concentrations as low as 50 mg/l. With high-performance liquid chromatography (HPLC), the purified material has proved to be a glycopeptide of 650 daltons. Its activity is removed by phosphate, or by certain peptidases and amylases. It binds to a lectin column and requires zinc for its effect. It loses activity on storage, but not if excluded from light and oxygen, or if kept with a small quantity of alpha-tocopherol, suggesting that its active form is that of the reduced zinc-peptide complex.

The structure of this glycopeptide has not yet been published, but analyses suggests that it may act in a manner analogous to the action of penicillin, by blocking bacterial cell wall synthetases during the manufacture of peptide-linked chains of muramic acid and N-acetyl glucosamine.

While the biological role of this remarkable substance may be to prevent the bacterial infection of HAF and the amniotic membranes, it seems likely that it also acts as an antiseptic, by cleansing the birth canal at the time of membrane rupture. Whether, by virtue of its human derivation, it will find a place as a therapeutic agent, remains to be seen. Work on its synthesis, tissue distribution and biological effects, and upon its relationship to the epidemiology of infection and amnionitis in pregnancy, is continuing and should prove interesting.

C. M. M. STERN
Department of Paediatrics,
St. Thomas’ Hospital,
London SE1 7EH, England

References