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Analytical Survey

Inter-laboratory transfer of HPLC methods: problems and solutions

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Abstract: As high-performance liquid chromatography (HPLC), the premier analytical technique in the pharmaceutical industry, becomes more ubiquitous, methods are more frequently being transferred from one laboratory to another. This review will discuss sources of failures to reproduce HPLC procedures, ranging from sample handling and preparation, through mobile phase, injector, column, detector and data manipulation problems. Also to be considered will be the precautions that should be taken, when initially developing a method, to obviate future problems. These precautions include using stable, well-defined analytical columns, buffered mobile phases, low wavelengths (or a mass-sensitive detector) and internal tests for accuracy; based on the author's experiences. Since the laboratory that originally developed the procedure has the moral obligation and, perhaps, the regulatory responsibility to "guarantee" that the method will perform successfully elsewhere, a series of increasingly comprehensive steps will be given, based on practice, to be followed by the laboratory that could not reproduce the procedure. Also to be discussed are approaches for treating methods that were initially successful but have slowly deteriorated and now fail, and several examples of procedures that were not reproducible in some other laboratories.

Keywords: *High-performance liquid chromatography; methodology transfer; method development precautions.*

Introduction

The rapid increase world-wide in high-performance liquid chromatography (HPLC) necessitates developing fool-proof procedures that can be transferred from one laboratory to another. This analytical survey article will discuss some sources of failure to reproduce HPLC procedures, the precautions that should be taken when initially developing a method to minimize future problems and how to manage problem assays. It will finish with an examination of several non-reproducible methods and how they were handled. This author considers the investigators who first issued the procedure to be responsible for its success long after publishing the method.

Instrument problems, like a clogged injector, a column with voids, a detector with a persistent bubble, a worn lamp or a maladjusted integrator, will not be considered.

Sources of Difficulties

Occasionally, differences in results are due to the written procedure being incomplete or ambiguous. In one method, the experimental instructions read "shake to help dissolve the sample". A mechanical shaker gave results averaging 10.04 mg (theory = 10.0 mg/tablet, relative standard deviation (RSD) = 0.9%) while sonication gave erratically low results averaging 9.4 mg with a RSD of 5%.

Sample handling

Frequently, what was considered to be a problem with the assay behaving oddly in different laboratories can be traced to the sampling. When full jars of 0.10% steroid cream formulations stored for 1–3 years at various temperatures were analysed, the results were within experimental error of theory. But when jars were only partly filled with samples and held for various periods of time prior to assay, results were as much as 10% above theory. Water, an excipient, evaporated from the partly-filled jars, as determined by gas chromatography. Correcting for water gave results for steroid content within experimental error of theory. Future samples will always be stored in small, tightly closed containers at 4° prior to analysis.

Inefficient sample extraction from a matrix is a frequent source of difficulty. One procedure required sonication with methanol for 15 min with occasional shaking of the volumetric flasks. Laboratory personnel that vigorously swirled the flasks averaged contents 3% higher than individuals who swirled the flasks only once.

Assays for contents of amitriptyline hydrochloride tablets gave results 5–10% below theory in one laboratory, due to adsorption of the analyte on the surfaces of a mortar and pestle. This electrostatic attraction frequently occurs with nitrogeneous compounds. No such loss was found after grinding tablets with a coffee bean grinder fitted with metal blades.

Mobile phase

Figure 1 shows that an impurity, formaldehyde, in one brand of methanol degraded the diuretic bendroflumethiazide [1]. So one laboratory using one brand or lot of methanol can obtain marked differences in results from another laboratory using a

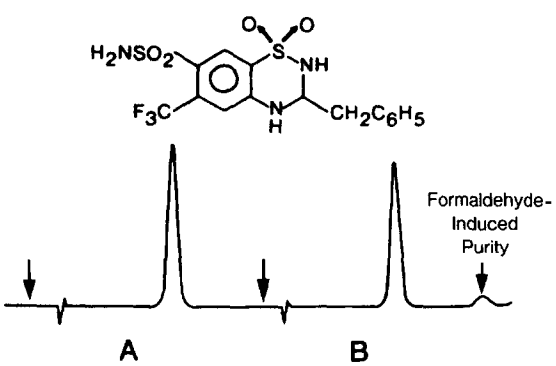

Figure 1
Chromatogram of bendroflumethiazide in (A) formaldehyde-free methanol from Baker and (B) methanol containing formaldehyde from Fisher. The phenyl column (Waters or ES Industries) was used with a mobile phase consisting of 0.1 M NaCl 0.025 M sodium acetate-methanol (60:40%, v/v). Detection was at 270 nm. Redrawn with permission of the *J. Chromatogr. Sci.* and Preston Publications, Inc.

different bottle of methanol as extraction solvent. The mobile phase should be prepared at room temperature, since some solvents, like acetonitrile, have a large coefficient of expansion with increases in temperature.

Dissimilarities in organic solvents used to prepare mobile phases can also produce laboratory-to-laboratory differences. Chloroform containing 0.5% ethanol as preservative has been known to elute compounds differently from chloroform without ethanol. Another preservative, butylated hydroxytoluene, which is sometimes added to tetrahydrofuran, causes differences, since this compound is both reactive and can introduce considerable ultraviolet (UV) absorption. The remedy is simple; use unstabilized tetrahydrofuran.

Silica columns were affected by varying concentrations of hydrochloric acid in different lots of methylene chloride used to prepare the mobile phase [2]. With time, the columns were destroyed by the methylene chloride-*n*-propanol mobile phase. Cyclohexene added as stabilizer can trap the hydrochloric acid but, in turn, could affect resolution.

Pump

Occasionally, interlaboratory differences have been traced to different pumps. For example, one laboratory used an isocratic pump to pressurize a mobile phase consisting of 98% aqueous and 2% organic solvents. Another laboratory used a gradient pump to blend these two constituents with low pressure mixing. Unfortunately, the error in the gradient proportioning valve was ±0.1%, causing a 5% error (98:2, 2% ± 0.1% = ±5%) in composition of the mobile phase, and resulted in the retention time of the analyte differing between laboratories. Another laboratory using another more accurate gradient pump might have negligible error, thus confounding the source of interlaboratory differences. Additionally, gradient pumps have been known to demix solvents to the extent of producting extra peaks in the chromatograms. These peaks could be mistaken for impurities [3].

On occasions, the pump cannot tolerate the mobile phase, even with so-called "hardened seals", which are more resistant to dissolution. Not only was the sample soluble in the mobile phase of 99.5% acetonitrile–0.5% water, but so were the pump seals.

Injector

Another vendor also manufactured an autoinjector that could not handle this acetonitrile solvent. This mobile phase ceased to be a problem when another make of autoinjector that could tolerate this acetonitrile-rich mobile phase was found.

The major interlaboratory problem introduced by autoinjectors is high dead volume. Figure 2 illustrates the difference between separations performed on a laboratory-assembled, modular LC system using low dead volume connecting tubing and the separation obtained on a commercial instrument containing both conventional, larger-diameter tubing, and excessive tubing in the autoinjector assembly. (In some instances, a high dead volume in an injector can be advantageous. If the injection solvent is not quite compatible with the mobile phase, the mixing of the two solutions may lead to improved chromatography.)

Columns

Differences between columns may be the greatest source of non-repeatability of

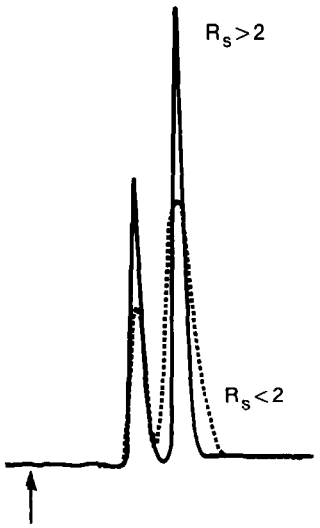

Figure 2
Comparison of the separation of identical constituents chromatographed on a modular LC assembled with minimal-length, low dead volume tubing (——) as compared with a commercial instrument containing ordinary connecting tubing (----).

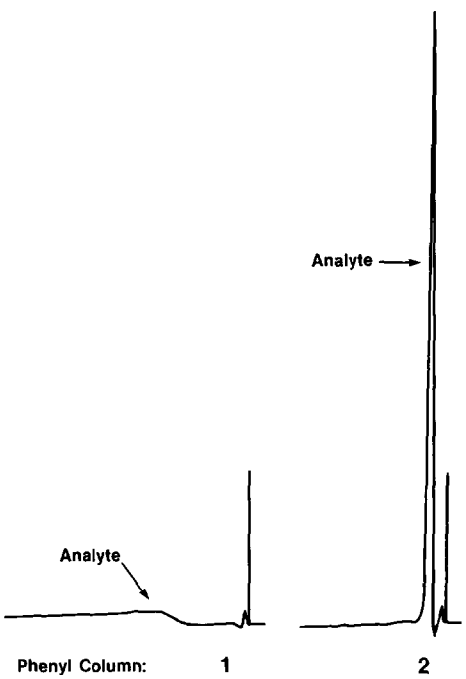

Figure 3
Chromatography of the same analyte using the identical chromatography parameters except for the phenyl columns being packed with two different lots of packing material. Flow is from right to left.

another laboratory's method. Figure 3 shows column-to-column variations using two phenyl columns packed with the same diameter of particles by the same vendor. The enormous differences between peak shapes under identical conditions are due to two different lots of packing being used. Silica columns from different vendors often give varying selectivities. Figure 4A illustrates similar, satisfactory superimposable, chromatograms using columns from either of two vendors, and Fig. 4B, four unsatisfactory separations due to either poor peak shape or resolution, using columns from four

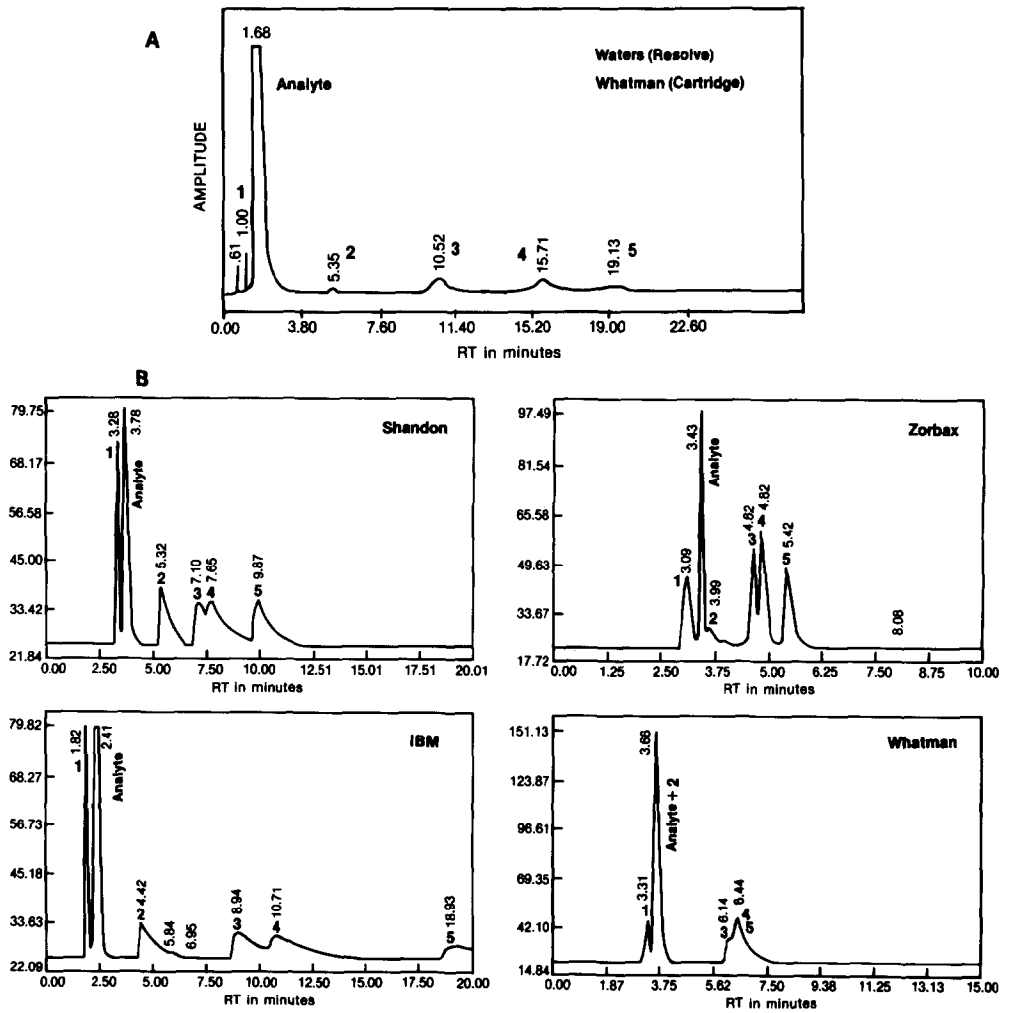

Figure 4
Comparison of silica HPLC columns using the identical, freshly-prepared test system. All other chromatographic variables were kept constant.

vendors; including two columns using different sizes of particles from one vendor. The identical, freshly-prepared test mixture was injected.

Peak reversals from one octadecylsilane (ODS) column to another are shown in Fig. 5. The identical pair of steroids was used. The difference may be due to the percent loading or coverage by the ODS. The 5% loaded column has a large number of silanol groups available to interact with the analyte, resulting in separations by both partition and adsorption. The 15% loaded column was endcapped to minimize such mixed-mode separations. Such reversals of elution order were noted previously, such as for the topical anti-inflammatory agent triamcinolone acetonide and its internal standard, fluoxymesterone [4].

Resolution of constituents can vary from laboratory to laboratory depending on the column. Figure 6 shows the differing resolution of 5 and 10 μm ODS columns from the

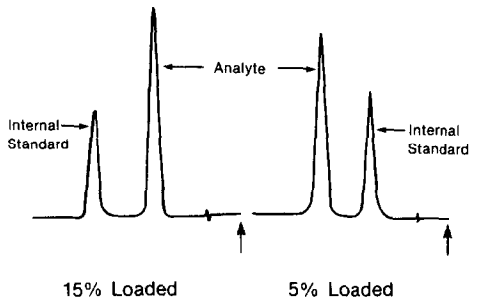

Figure 5
Reversal in elution pattern of two steroids using 5 and 15% loaded ODS columns. All other chromatographic parameters were held constant.

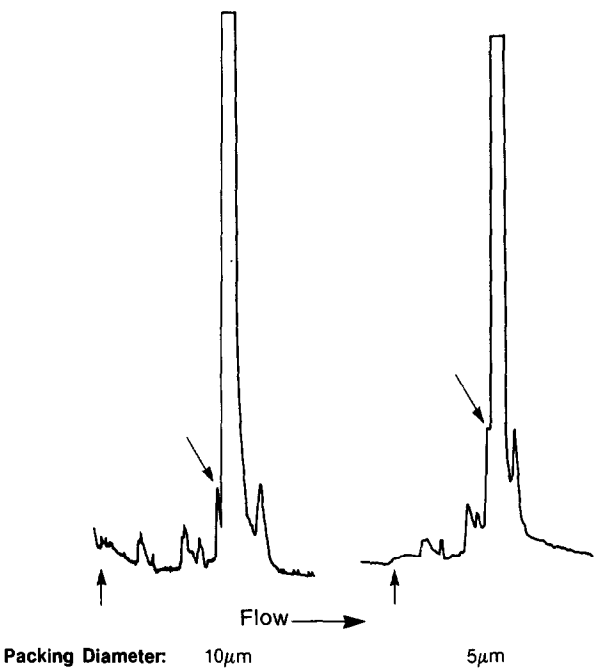

Figure 6
Differing resolution of 5 and 10 μm, 5% loaded ODS columns. Note that the 10 μm filled column resolves two particular closely-eluting components better, but, in general, resolution is superior with the 5 μm column.

same vendor. This figure was chosen to illustrate the point that selectivity can vary between ostensibly similarly treated particles. Another example of resolution depending on the column is shown in Fig. 7. In this situation, the extra peak resulting from degradation of an excipient with time and temperature was resolved from the steroid analyte on one octadecylsilane column but not on another. The effect of such an unresolved peak will be to increase the area of the sample as compared with the standard; i.e. the results will be above 100% of theory. The peak height of the sample may be unaffected by the contaminating constituent if it elutes sufficiently distant from the point of greatest height. Thus, two laboratories will obtain different or similar results, depending on the type of response measurement as well as the column chosen.

A once-satisfactory column, as it ages, can yield different results. For bonded phases, resolution and retention times usually decrease [5] with time.

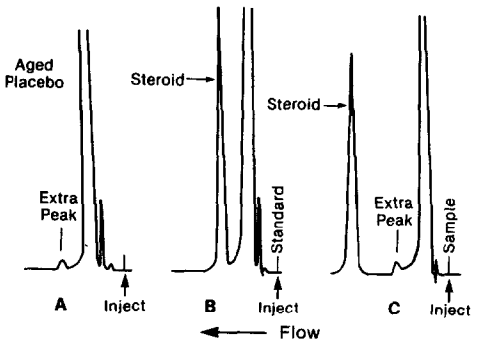

Figure 7
A: HPLC using one ODS column of an extract of aged placebo formulation showing an extra peak due to degradation of an excipient. B: an extract of active formulation chromatographed on the same ODS column lacking the selectivity to resolve the constituents. C: a chromatogram of the extract on a different ODS column that can resolve the analyte from the excipient impurity.

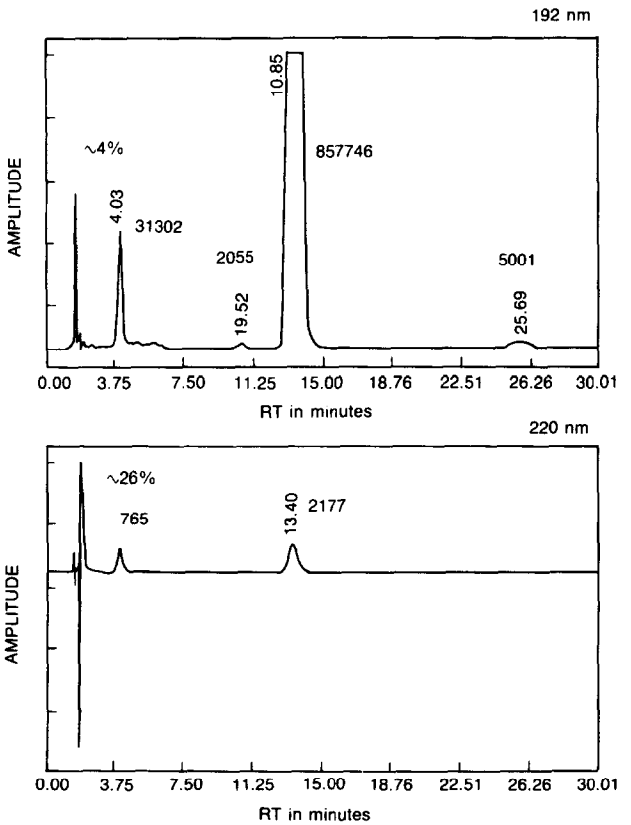

Figure 8
Dependence of apparent impurity content on detector wavelength. Note that the apparent impurity content depends on the wavelength.

Detector

Since there are many vendors and models of variable wavelength detectors, the design aspects may affect resolution. For example, one detector had a dead volume for the fitting of 24 μl and a flow cell volume of 14 μl, R_s = 1.3. With a fitting dead volume of 0.5 μl and a flow cell volume of 12 μl, R_s = 3.4. Figure 8 shows how for one compound, the apparent impurity content depends on the wavelength chosen. The difference of

18 nm in detector wavelength gave a difference in apparent impurity content of 24% for an average of 1.3% nm^{-1}.

Since an uncalibrated detector easily could be 2 nm distant from the true wavelength, a 3% difference in apparent impurity content would be found between two laboratories. Normally, a relatively flat region of the UV spectrum is chosen for the detection wavelength. Sometimes a mass sensitive detector is used. More frequently now, a diode array detector is used to examine ratios of absorbances at different wavelengths to avoid such problems. Other interlaboratory problems arising from detectors include the inability of one detector to visualize an impurity due to lower sensitivity than the one used to develop the method (especially important with fluorescent and refractive index detectors where there is a wide variety of sensitivities), different band width characteristics, different susceptibilities of electrochemical detectors to electrode inactivation by an impurity and trace impurities in one laboratory contaminating a fluorescent detector, which may produce high readings.

Data reduction

Different integrators or integration parameters can produce different results in other laboratories. Figure 9 illustrates several problems. There is the frequent problem of integrating a small, incompletely-resolved, peak. Depending on the integration parameters chosen, different areas might be found. Depending on the sophistication of the integrator, if there is baseline drift up or down, the calculated content of the late eluting impurity could be lower or higher than the actual value. Modern integrators can either sense and readjust integration parameters to accommodate drift or permit manual setting of parameters to overcome drift-induced problems. It is easy to mistake a system peak [6] arising from the mobile phase, as an impurity. Of course, the peaks must be correctly designated, which in complex systems (such as 40 component amino acid separations [7]), may be difficult, especially if there are retention time shifts due to a matrix effect.

Results should be reported using unambiguous terms. For example, ion chromatography of a 50 ppm standard gave 10 area units. A 1 mg ml^{-1} sample yielded 2 area units. But the content of ion in the sample should not be reported as 10 ppm (based on a

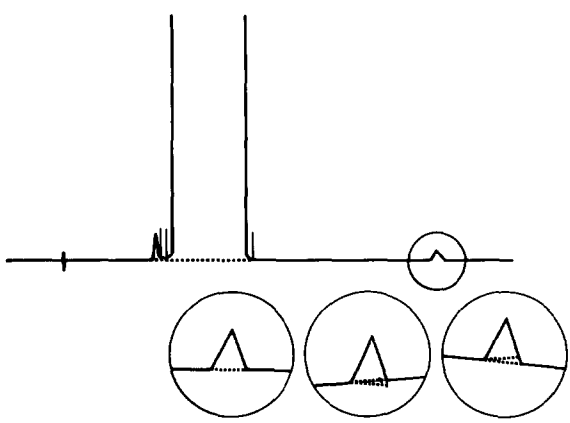

Figure 9
Composite chromatogram illustrating the problems of integrating incompletely resolved peaks and late-eluting peaks when the baseline drifted.

response per ml calculation). Based on 1 mg of solid in solution the content was 10,000 ppm.

Precautions to be Taken when Developing HPLC Methods to Minimize Future Problems

Table 1 lists the precautions this author finds should be taken when devising HPLC assays. Although several of these appear elementary and obvious, this investigator has anecdotal evidence that failure to observe them has created embarrassing difficulties. Defining sample handling responsibilities when personnel from more than one laboratory are involved, the first precaution, is intended to prevent the accidental omission of steps because each person believed that the other carried out the task. Erratic results have been generated in one laboratory because no one mixed thawed samples in the mistaken belief that the individual who removed the samples from a freezer located elsewhere had shaken the samples prior to delivery.

Using reasonable sample sizes usually is self-evident. Unless there is a shortage of material or it is expensive, use 20 mg to as much as 100 mg sample of bulk or pure material to increase accuracy (especially if inhomogeneity may be a problem). The final injected solutions, of course, should be sufficiently dilute to avoid exceeding the linear range of the detector. Using excess solvent gives the leeway needed when the laboratory may be cold, and solubilities in chilled solvent are lower, or when the analyst did not shake the flasks quite as vigorously as when the method was first developed and validated. For example, Table 2 shows that two extractions of steroid using acetonitrile/

Table 1
Precautions to be taken when developing a HPLC method

Define sample handling responsibilities when personnel from more than one laboratory are involved
Use reasonable sample sizes and volumes
Use excess solvent for sample
Test effect of solvent on analyte
Test effect of excipients on analyte
Use a stable LC column
Buffer the system
Use mobile phase as injection solvent
Build accuracy tests into the method
Know the effect of changing parameters
Develop an alternative method for important assays
Test if impurities have same spectral properties as the analyte if using a spectrophotometric detector
Be prepared to isolate impurities for structure elucidation
Question if the results are logical and reasonable
Verify method in another laboratory
In report or paper, emphasize any key steps or precautions
Anticipate special interests of recipients of the method

Table 2
Total percent steroid extracted

Steroid extraction	% Steroid extracted
1	95.2
2	99.7
3	99.8
4	99.9

water (2:1) from the formulation in hexanes are sufficient to give passable results, and that three extractions should suffice. However, the fourth extraction ensures that quantification will be almost always uniform from person to person. Naturally, if 12 ml of solvent are needed to dissolve analyte, the "even" or "round" quantity of 25 ml should be used to provide for total dissolution even when the solvent is cool. The use of a small laser to test for the Tyndall effect produced by suspended analyte is recommended.

The analyte should be tested for reactivity with solvent. Figure 10 depicts multiple pathways of steroid degradation by methanol [8]. Such testing also yields the time limit that samples can remain in autoinjector vials (either at ambient temperature or 4°) prior to injection.

Extenders, diluents and excipients can also react with the analyte. The upper portion of Fig. 11 depicts the chromatogram resulting from the extraction with methanol of a trial tablet formulation of a phosphoric acid ester antihypertensive agent and the lubricant magnesium stearate. The ester was hydrolysed by the magnesium salt; cf. the lower portion of Fig. 11. The molar ratio of stearate to ester is 1:1 in a model system. When

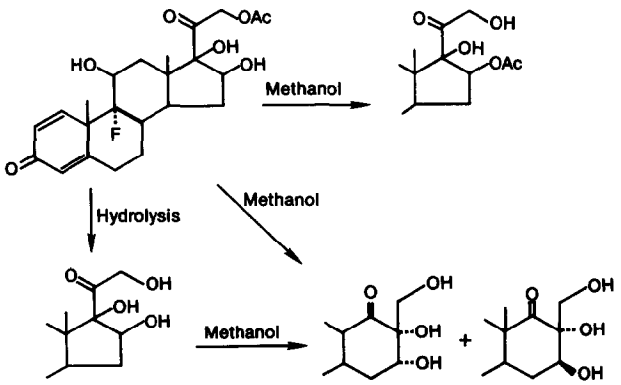

Figure 10
Effect of solvent on the analyte: methanol induces transacetylation and D-ring rearrangements of a 19-acetate and its hydrolysis product.

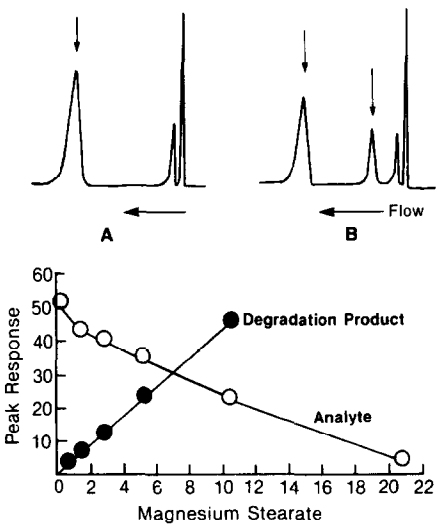

Figure 11
Upper portion: HPLC of a phosphoric acid ester using a 5-μm ODS column and a mobile phase of methanol/aqueous 0.2% phosphoric acid (72:28% v/v) with detection at 214 nm. A, in methanol with magnesium stearate absent; B, in methanol with magnesium stearate present, showing the appearance of a new peak and a lower response for the analyte. *Lower portion*: degradation of the ester analyte (○) to the hydrolysis product (●) as a function of the content of magnesium stearate in methanol. The lower scale shows time in hours. Redrawn from *J. Chromatogr. Sci.* **23**, 493–498 1988, with permission of the authors and the Preston Publishing Company.

water was used as the extracting agent, negligible hydrolysis of drug was found because of the poor solubility of magnesium stearate in water. Thus, the stability of the analyte was dependent on the extracting solvent well as the presence or absence of an excipient.

LC columns are susceptible to change. Cyano groups can hydrolyse to carboxylic acids, poly(styrene-divinylbenzene) columns can oxidize, amino columns can react with oxo groups to form Schiff bases, ions can contaminate anionic and cationic exchangers, and hydrocarbons can contaminate reversed-phase columns resulting in changed selectivities. Guard and saturator columns provide significant protection. When initially developing a HPLC method, a 15% loaded, endcapped ODS column is preferred for its relative stability. (If repetitive injections yield changing peak shapes, covalent or non-covalent reaction with the column is indicated [9].)

Columns should be used in a buffered environment, in addition to buffering the mobile phase against changes in pH. Organic mobile phases should have a small quantity of water added to buffer against slow water absorption from the air; 0.5–1.0% water usually is sufficient. Placing the column in a thermostatted chamber, maintained several degrees above ambient, can halve the RSD of repetitive injections spanning 12 h or more.

The injection solvent should be mobile phase to avoid extra peaks and peak anomalies. Figure 12 depicts the marked effect on peak shape of hydrochlorothiazide using a solvent dissimilar to the mobile phase. As discussed previously, the analyte should be tested for stability in the mobile phase, for as long as the sample may be dissolved prior to assay or reassay. Where this is not practicable, use a solvent with a weaker eluent than that of the mobile phase. Avoid mobile phases having very low concentrations of second or third components.

Too short a dissolution time prior to injection can also produce anomalous peaks. If the analyte is not totally dissolved, multiple peaks or trailing, significantly asymmetric peaks may appear [9].

During method development, gradient elution should be used to search for long-retained constituents. Naturally, the more important the assay, the more time should be devoted to it, even if it appears to be trouble-free. For example, results should be

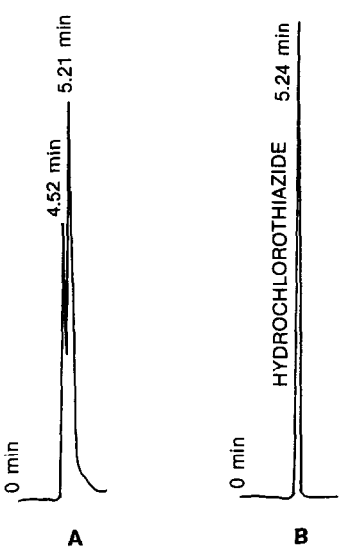

Figure 12
Chromatograms of hydrochlorothiazide dissolved in (left) acetonitrile and (right) mobile phase, using a RP-18 column with a mobile phase of acetonitrile/water/acetic acid (18:81:1%, v/v/v) at 1.0 ml min^{-1}. Detection was at 275 nm. Figure courtesy of T. L. Ng and S. Ng, *J. Chromatogr.* **329**, 13 (1985), Elsevier Science Publishers, Amsterdam.

verified by using an alternative method like TLC or another HPLC system having different polarity [10]. This is especially useful in revealing unresolved impurities. A diode array detector should be used to ascertain peak homogeneity and, if impurities are visible, the molar absorptivity of the analyte should be compared with that of the impurity. Response factors of known impurities should be ascertained.

Assay results should always be logical and self-consistent. A 5% impurity content is inconsistent with a 99.8% purity value. The analyst has the task of ascertaining if the impurity has a high molar absorptivity, is a highly absorbing, contaminating solvent or if the sample was measured against a standard assigned an erroneously high purity. Some analytical problems can be avoided if the assay procedure contains a daily, built-in test for accuracy, in addition to the initial method validation and ruggedness assessments. As described in Table 3, such a test for accuracy involves two standards containing either a native or an added impurity. Using two weighings of standard from two vials, one solution is analysed for purity against the other to test the LC system and integrator. The impurity content is used to test for resolution and quantification. Peak height results should agree with those obtained by peak area. Reasonable limits to system suitability parameters (such as resolution factors and retention times) may have to be set.

To ensure that a method works, send a copy of the procedure to another laboratory, preferably the receiving laboratory, for comments and to verify the assay. This is a good test for finding transient, but essential, punctuation and ambiguous directions. For example, using the direction "dilute", 40 ml of water diluted to 100 ml with methanol gave a k' for toluene of 2.32, whilst 60 ml of methanol diluted to 100 ml with water yielded a k' of 3.05 [11].

The last precaution involves some knowledge of the idiosyncracies and special likes and dislikes of the people who will be scrutinizing the final report and, perhaps, duplicating the method themselves. Cater to them, especially if the extra time needed to supply the special data that they want is minimal. Perhaps they are right.

Table 3
An injection sequence to test for accuracy

Standard 1	Discard first injection
Standard 1 Standard 2	Analysis of one standard against the other should give value for purity within 1.5% of theory. Impurity content must agree with prior results
Impurity standard Sample 4 Sample 1 Sample 5 Sample 3 Sample 2	Randomized samples
Standard 2	
Sample 5 Sample 2 Sample 4 Sample 1 Sample 3	Computer monitors areas of each standard for constancy, k', R_s or analyte, number of theoretical plates, peak asymmetry, number and location of peaks, and, if present, impurities
Impurity standard Standard 1 samples Standard 2 samples	

How to Handle Problem Assays

Since everyone eventually has to grapple with problem assays, it should be noted that regardless of the source of a failed method, the remedies are essentially similar. As summarized in Table 4, undesirable results should be discussed between method donor and recipient. The possibility that the sample is the source of the difficulty can be eliminated by exchanging samples. The analysts should examine each other's chromatograms, integrations and calculations. Then a column that worked successfully in one laboratory may be transferred to the laboratory having problems. If needed, and feasible, the mobile phase may be transferred, and the chromatography components. If necessary, the analyst may be transferred; the person who does the work and not a supervisor or manager. If all else fails, the method will have to be redeveloped because a one-laboratory method is intolerable.

Table 4
How to handle a problem assay (either as donor or recipient)

1. Discuss difficulties calmly, using chromatograms, calculations, etc.
2. Discuss method to ascertain if it was followed without change
3. Reassay some samples in both laboratories to verify if procedure is faulty
4. Transfer a tested column
5. Transfer a tested mobile phase
6. Transfer, if possible, other equipment
7. Transfer an analyst
8. Redevelop the method

Occasionally, an assay is satisfactory for long periods of time and then it fails. Analytical columns change with use, and column manufacturers can create large differences in selectivity by ostensibly minor changes in fabrication.

The differences may be due to a change in synthesis of the compound or manufacturing of the matrix for the analyte. In these instances, the analyst must look for reasons, questioning the submitter and hoping for answers. Too often, the initial responses are usually similar to "Nobody knows nothing" and "You know, you never know, You know?" Further questioning frequently reveals useful information.

Some samples change upon ageing. As shown in Table 5, the analyte was stable; the cause of the apparently lower results was that the original extractions of the analyte were unsuitable and had to be redeveloped to cope with the aged samples. This is also an instance of the analyst simultaneously being hero and villain.

Examples of Assays That Were Non-reproducible in Other Laboratories and How These Situations Were Managed

Finally, several examples of procedures and methods that gave considerable difficulty when transferred between laboratories, are discussed.

Excellent linearities for the peak areas and heights of the anti-hypertensive agent, captopril, in several injection solvents were found [12, 13] using ODS columns from several vendors (Whatman, ES Industries and Waters Associates), a mobile phase of methanol/water/*o*-phosphoric acid (25:75:0.05 to 60:40:0.05%, v/v/v), a 20 μl constant injection volume precision loop injector or autoinjector, and a variety of variable

Table 5
Analyte contents of a 0.10% formulation changing with time and thus necessitating different sample preparation methods (the final assay using HPLC remained unchanged)

Method	Time formulation stored (months)	Temperature	Percent (theory = 0.10) ($n \geq 2$)	Comments
1. Original extraction of analytes plus excipients	0	—	0.099	
	3	25°	0.100	No apparent degradation products
		40°	0.097	
		50°	0.096	
2. Modified extraction	3	all	0.099	Average
	4	25°	0.097	
		33°	0.052	No apparent degradation products
		40°	0.095	
		50°	0.097	
3. Remodified extraction	4	all	0.100	Averaged
	5	33°	0.094	No visible degradants
		40°	0.095	
4. Total solution of analyte plus excipients	5	33°	0.099	
		40°	0.099	
		50°	0.099	
	9	25°	0.100	
		33°	0.100	
		40°	0.100	

wavelength UV detectors (Hitachi Model 100-30, Kratos Models 770, 773 and 783, and Perkin Elmer Models LC-75 and LC85) set to 214–220 nm. In contrast, Berridge [14] found non-linearity of both peak areas and heights (Fig. 13A) using a Hewlett–Packard Model 1090A liquid chromatograph equipped with a diode array detector. We repeated his study using a variety of detectors and his identical conditions but for a 15 cm rather than 10 cm ODS columns. With a Kratos Model 783 detector, linear range 0–3 AUFS, excellent linearities were found, with correlation coefficients >0.9999 for both peak height and peak area responses versus concentration. A Waters Model 481 detector, linear range 0–2 AUFS according to the vendor, gave a correlation coefficient of 0.99997 for peak area but 0.9953 for height (Fig. 13B). Using a Hewlett–Packard 1040M diode array detector, which was similar to the detector in the 1090A LC, linear range −0.2 to 1.5, we found severe plateauing of the peak area responses (coefficient of correlation, 0.936). We concur with Chan and Yeung [15] that the detector should be operated in the linear range to achieve linear responses. In addition, the geometry of the LC system (permitting more or less diffusion of the peak) and detector band pass widths are important. We found linear responses of peak areas with varying concentrations (correlation coefficient, 0.99967) using a 4 nm band pass. This linear response is dissimilar to the non-linear area responses found by Berridge (Fig. 13A), and once again demonstrates the difficulty in exactly repeating work performed in another laboratory. (Variable volume injections may also contribute to non-linearity.)

We also reported that several compounds gave different peak areas and heights after being dissolved and injected in different solvents, like water and methanol. Other HPLC conditions were held constant. Chan and Yeung commented [15] that one such

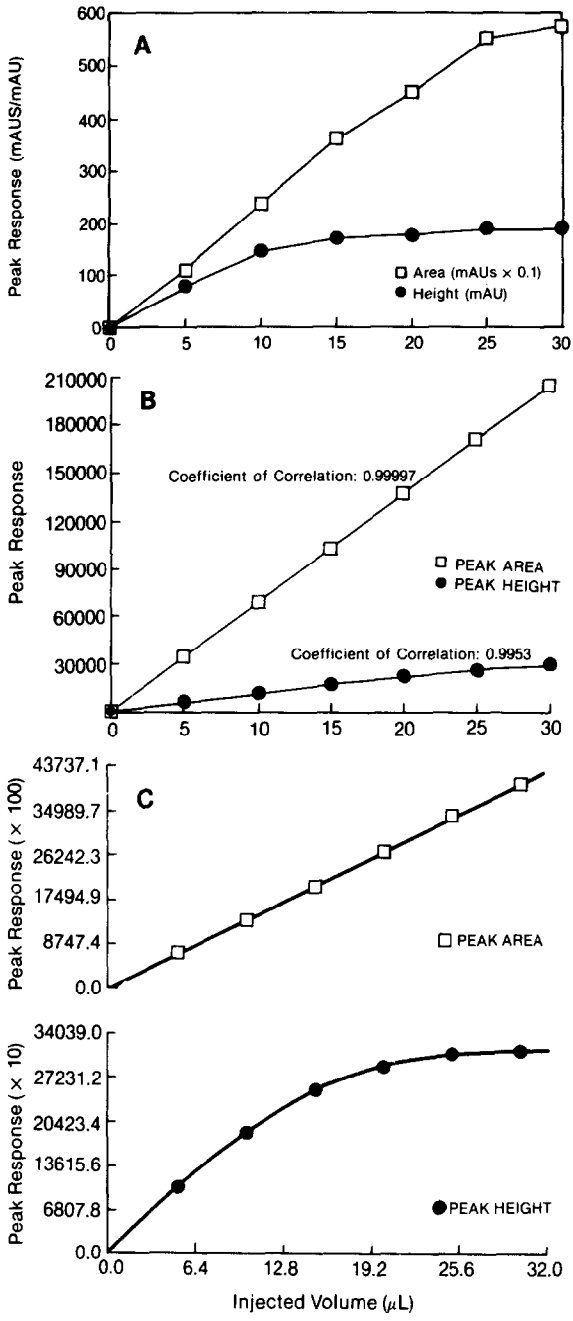

Figure 13
HPLC of captopril in methanol at various concentrations injected in LC under similar conditions, using various UV detectors; A, Hewlett–Packard diode array detector (1090 A System); B, Waters Associates Model 481 detector; and C, Hewlett–Packard 1040 A diode array detector. Part A was redrawn from *J. Chromatogr.* **369**, 265–268 (1986), with permission of the author, Dr J. C. Berridge and Elsevier Science Publishers.

compound, aztreonam, eluted in the void volume and thus the differing peak responses were an artifact. The varying peak responses found for aztreonam were reinvestigated using analyte dissolved either in acetonitrile/0.1 M ammonium phosphate buffer, pH 2.0 (75:25%, v/v; mobile phase) or water, and chromatographed on a diol column (ES Industries; Fig. 14). The capacity factor is 0.62, and two possible impurities were found to elute prior to aztreonam, eliminating a void volume phenomenon being responsible for the different peak areas of 36% between the two injection solvents. Gradually decreasing the acetonitrile content of the injection solvent to 70%, decreased the peak area responses in a linear fashion.

The inability of Chan and Yeung to detect differences in the UV peak responses generated by captopril dissolved in either water or methanol led to a collaborative study involving several independent laboratories. Figure 15 shows the dissimilarities found in one laboratory. In contrast, another laboratory using a manual Rheodyne 20 μl fixed loop injector, a 10% loaded ODS column (Whatman, Inc.) and a Kratos 773 detector, reported similar responses for captopril dissolved in methanol versus captopril dissolved in water injected shortly after dissolution and 20 h later. Dissolution with or without the aid of sonication and switching columns gave similar results. We can only conclude that as of this date, the phenomenon of captopril exhibiting different peak responses in differing solvents is apparent in three laboratories of four. However, thanks to the public nature of this controversy, other investigators forwarded related data to me.

Dr Malcolm Robinson (personal communication) sent data summarized in Fig. 16. Figure 16A is a chromatogram of 0.10 mg ergosterol dissolved in chloroform compared with an injection of chloroform (blank). Figure 16B shows the responses of a constant ergosterol concentration in various concentrations of methanol added to chloroform used to dissolve the steroid. A silica column, 250 × 4.6 mm, 5 μm (Whatman, Inc.) was used with a mobile phase of glacial acetic acid (0.5%), THF (1%), methanol (3%), dichloromethane saturated with water (30%) and dichloromethane (to 100%). Detection was at 280 nm. Retention times remain the same although methanol induced diminution of the peak area.

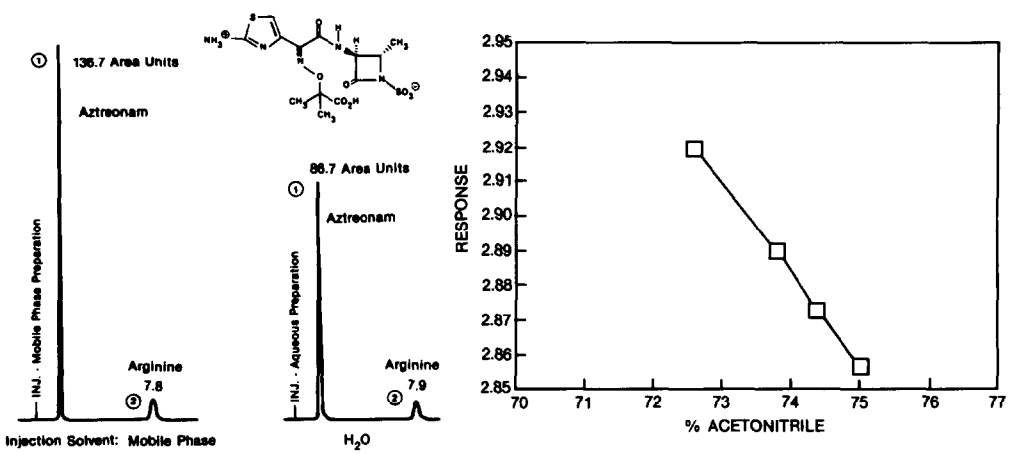

Figure 14
Left: HPLC of 300 μg aztreonam and arginine dissolved in either aqueous acetonitrile (75%)–0.1% phosphoric acid (25%) (mobile phase) or water and chromatographed on a diol column (ES Industries). Detection was at 206 nm. *Right*: Effect of varying the acetonitrile concentration on the peak area responses of aztreonam.

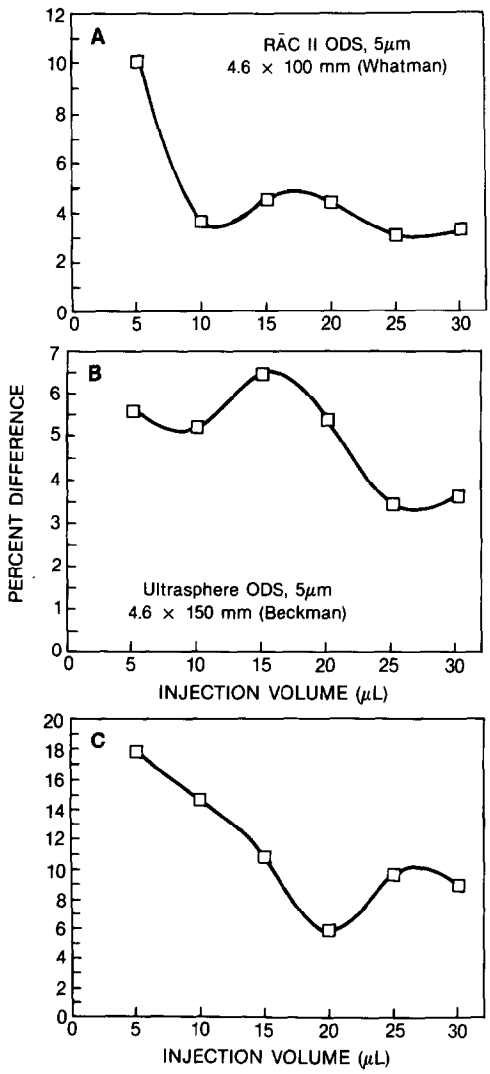

Figure 15
Differences between UV area responses of captopril dissolved in either water or methanol (0.8 mg ml^{-1}) plotted as percent, to eliminate the effect of differing injection volumes by using normalizing area counts. Mobile phase, methanol/water/0.05% phosphoric acid (50:50:0.05%, v/v/v) flowing at 1 ml min^{-1}. A, RAC II ODS column, 100 × 4.6 mm, 5 μm (Whatman), Kratos 783 detector; B, Ultrasphere ODS column, 150 × 4.6 mm, 5 μm (Beckman), Kratos Model 783 detector; and C, Ultrasphere column, Hewlett–Packard 1040M detector. The wavelength was 214 nm for all experiments.

Figure 17 is redrawn from data published by Dr N. Parris. The peak areas and heights for pyrene depend, in part, on the injection solvent using a 250 × 2.1 mm ODS column (Zorbax), and a mobile phase of methanol/water (4:1%, v/v) following at 0.25 ml min^{-1}. Injection volumes were 10 μl. Although the original data are unavailable, retention times appear similar by visual examination. Ratios of peak area/height are 5.1–6, indicating some similarity of the effect of the injection solvent on the pyrene peak responses.

The possible importance of this solvent-dependent peak response phenomenon to analytical chemists is summarized in Fig. 18. Here, identical weights (25 ± 0.02 mg) of two compounds gave ~3% different responses after being dissolved and injected in methanol (from a freshly-opened bottle) and methanol from the identical bottle to which was added the equivalent of 1% water. The two solutions were injected onto the identical phenyl column (ES Industries) using a mobile phase of methanol/water/*o*-

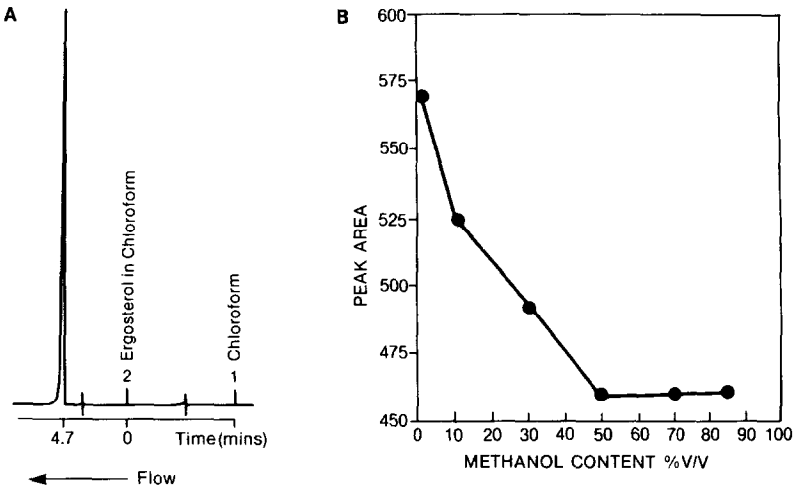

Figure 16
Effect of varying the methanol content of chloroform–methanol injection solvent for ergosterol. A, HPLC of 0.1 mg ml^{-1} ergosterol in chloroform and chloroform alone. B, Effect of methanol on peak area responses of ergosterol dissolved in chloroform. A silica column, 250 × 4.6 mm, 5 μm (Whatman, Partisil) was used with a mobile phase of glacial acetic acid (0.5%), THF (1%), methanol (3%), methylene chloride (water-saturated) (30%) and methylene chloride (to 100%) flowing at 1 ml min^{-1} into a detector set to 280 nm.

phosphoric acid (45:55:0.05%, v/v/v) flowing at 1.2 ml min^{-1}. Peak area and height responses are shown adjacent to each peak. If the two analytes in methanol were quantified versus the two in aqueous methanol (without replicates) the 3% differences could either be ascribed to experimental error or reported as being borderline significant, since a 3% experimental variation is possible. If one constituent was analyte and the other in the mixture internal standard, the ratios would be similar to the responses in the other injection, and no difference in content would be calculated. Thus, alternative experimental designs could reveal different conclusions; either methanol containing water absorbed from the atmosphere induces a different response (or responses) versus dry methanol or there is no difference. This investigator urges that injection solvents be similar, despite the common practice for biological samples being extracted, the extract evaporated, and the residue resolved in one solvent and then quantified versus standard in a dissimilar solvent. In another pharmaceutical manufacturing company, a formulation gave 4–5% greater peak area responses, in two laboratories, than the standard, until acetate was added to the standard (Eugene Inman, Lilly, personal communication) [16], showing that solvent disparities can affect apparent drug contents.

Another example of an irreproducible method was a procedure that functioned properly over an extended period in the originating laboratory. It was a HPLC assay using low wavelength UV detection of an organic ion in a complex pharmaceutical preparation. However, a second laboratory reported unstable baselines, poor peak shapes and an insufficient limit of detection. A third laboratory was given the written description of the method and asked to reproduce the assay independent of the other laboratories. The results obtained were satisfactory and the method judged free of difficulties and rugged. Unfortunately, the roller coaster ride continued with a fourth

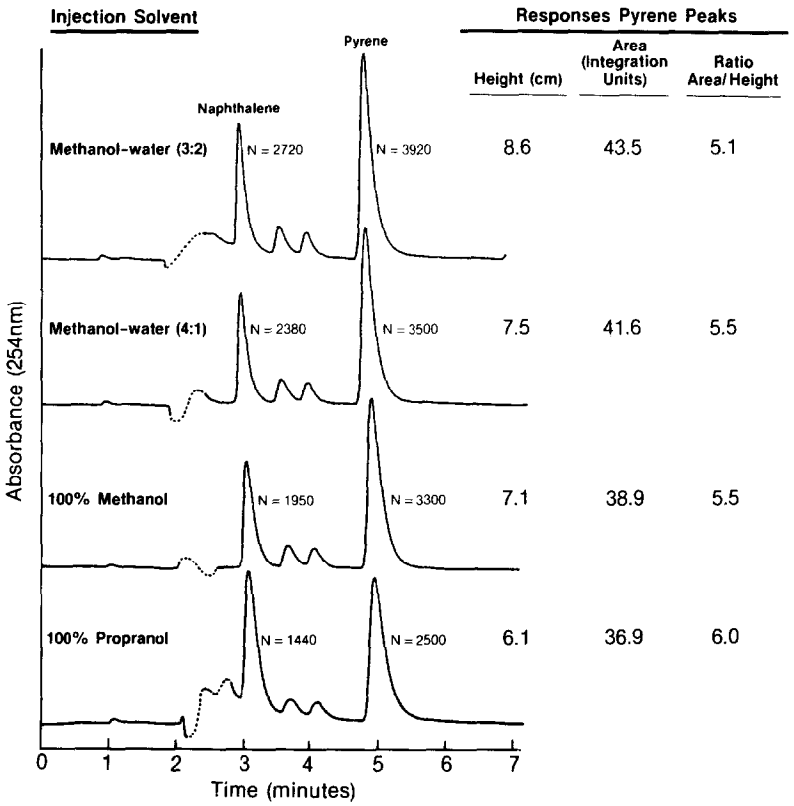

Figure 17
Effect of various injection solvents on the HPLC responses of pyrene. A Zorbax ODS column 250 × 2.1 mm, 6–8 μm (duPont) column was used with a mobile phase of methanol/water (4:1) flowing at 0.25 ml min^{-1}. Injection volume: 10 μl. Redrawn from N. A. Parris, *Instrumental Liquid Chromatography*, p. 231, with the kind permission of the author and Elsevier Science Publishers, Amsterdam.

laboratory complaining that the columns, all of which came from the sole vendor, had to be tested for suitability prior to use and that the few satisfactory columns had a short lifetime. The variety of problems encountered in two laboratories led to the method being redeveloped, since two experienced chromatographers found the assay to lack ruggedness.

The final example of a HPLC method that gave different results in different laboratories involved an assay for two analytes using a silica column with a reversed-phase type of aqueous acetonitrile mobile phase. Although the analysts in the laboratory that originally developed the method had few problems with the procedure, another laboratory reported poor resolution and asymmetric peak shapes. A referee laboratory showed that almost all silica columns were unsuitable, and the assay was judged too fragile for general use. Because an investigator in an independent laboratory successfully utilized this method, apparently unaware of its erratic nature, he was asked about difficulties. He reported that the major problem was that the column required overnight equilibration with mobile phase. Thus, one observant analyst was able to identify a second variable (in addition to several vendors' columns being unsuitable) causing irreproducibility of a HPLC method in different laboratories.

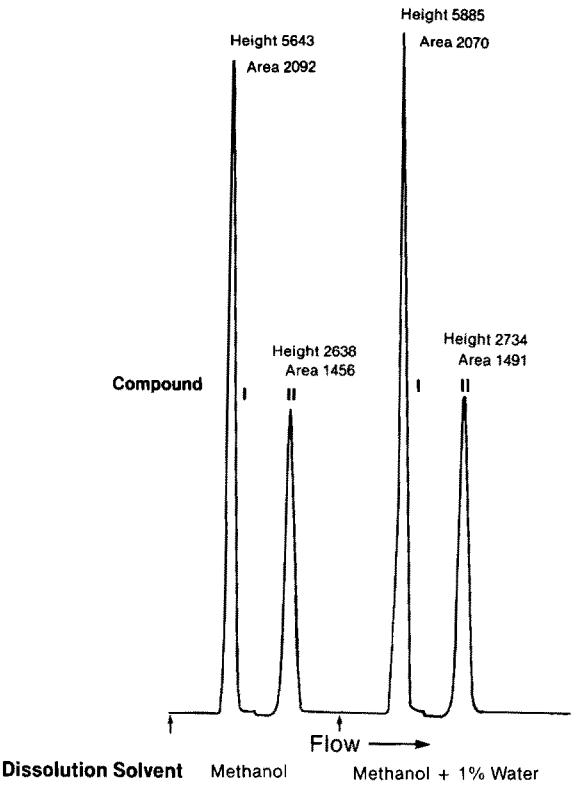

Figure 18
HPLC of two substances dissolved in either dry methanol or methanol containing 1% added water prior to injection. Both peak areas and heights differ.

In summary, because HPLC is the premier analytical technique, care must be taken to develop rugged and reproducible procedures. This paper discusses a variety of causes of irreproducible methods, precautions to be observed while developing methods and illustrated techniques for handling problem assays.

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