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Chemometrics in pharmaceutical analysis*

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Abstract: Pharmaceutical analysis is undergoing a slow revolution as chemometric principles become increasingly incorporated. This paper reviews some of the more recent advances, with particular focus on spectrophotometry, chromatography and expert systems.

Keywords: *UV spectrophotometry; liquid chromatography; pharmaceutical analysis; expert systems.*

Introduction

Pharmaceutical analysis embraces all aspects of the in-process and quality control testing of drug substances and their formulated products together with their physico-chemical and stability assessment. Through judicious application of chemometrics, there is a slow revolution taking place in pharmaceutical analysis as mathematical, statistical and computer-based principles become incorporated into the analytical laboratory. A recent review [1] considered some of the challenges that face the pharmaceutical analyst particularly when developing new chromatographic methods, undertaking stability studies, or introducing automation into the laboratory, and how chemometrics is impacting upon these activities in an extensively regulated environment. However, both regulatory agencies and chemometrics have moved forward and in this review we consider recent advances which have been made in the application of chemometrics in pharmaceutical analysis, particularly in spectrophotometry, chromatography and with expert systems.

Spectrophotometry

The pharmaceutical analyst has long made use of UV–vis, infrared (IR) and to a lesser extent fluorescence spectrophotometry. More recently diode array spectrophotometers have revitalized UV–vis spectrophotometry and interest is increasing in the applicability of near infrared (NIR) spectrophotometry in pharmaceutical analysis. UV–vis spectrophotometry is a simple, rapid technique which has suffered in its range of applicability due to a lack of selectivity. Chemometrics provides an ideal means of extracting quantitative information from the spectra of multicomponent samples.

A recent study [2] on the simultaneous determination of nifedipine and its oxidation product, 4-(2-nitrosophenyl)-2,6-dimethyl-3,5-dicarbomethoxypyridine, may be regarded as the simplest form of chemometrics. Measurement or determination of the molar absorption of the two components at two separate wavelengths enables quantification of those components in an unknown sample by solution of the requisite simultaneous equations. The two wavelengths chosen in the nifedipine study [2] provided an exactly-determined system in accordance with the criteria embraced by Smeyers-Verbeke *et al.* [3]. The latter demonstrated that over-determination gave no better accuracy and precision. The application of this approach to a ternary mixture of meclozine hydrochloride, pyridoxine hydrochloride and caffeine has recently been reported [4]. Factors affecting the accuracy and precision were discussed; the study concerned being aided by the ability to determine pyridoxine directly at 307 nm without interference from the other components.

Derivative spectroscopy has been used to sharpen absorbance bands and to remove excipient interferences. Electronic differentiation modules have been used in the past but

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the proliferation of diode-array spectrophotometers, with the attendant storage and processing facilities, has provided the ideal hardware/software configuration for derivatization of the digitized spectrum. Although differentiation increases selectivity by reducing the spectral bandwidth, the signal-to-noise ratio is also reduced so cumulative measurements are required to recover precision.

The influence of derivative order and analytical wavelength range on precision in the determination of paracetamol and codeine in combination tablets has recently been investigated [5]. The results showed that, for signals of comparable intensity (300 mg paracetamol, 60 mg codeine), differentiation merely lowered the precision, whereas when the components gave signals of disparate intensity (300 mg paracetamol, 30 or 15 mg codeine) the first order spectra gave optimal accuracy and precision. This was in large part due to the choice of wavelength range and poor signal-to-noise ratio. The authors noted anomalies in their higher order spectra; probably as a result of problems with instrumental sensitivity which resulted in unsatisfactory analysis of tablets containing a combination of 300 mg paracetamol and 7.5 mg codeine. The use of second derivative spectra and an instrument of superior performance may well give satisfactory results.

The Kalman filter, a recursive comparison of experimental data with a mathematical model, has been applied recently to the simultaneous UV determination of sulphamethoxypyrazine and trimethoprim in tablets [6]. Use of the filter gave results comparable with dual wavelength determination but with less variance for replicate determinations. The advantages and potential problems are illustrated by the determination of dextromethorphan, triprolidine and pseudoephedrine. Kalman filter software has been incorporated into a commercially available spectrophotometer [7]. Whether the Kalman filter finds more universal utility will be dependent upon finding a validation protocol acceptable to regulatory agencies.

A technique for quantification of components in multicomponent UV–vis spectrophotometry that has received little attention from pharmaceutical analysts is fuzzy theory. For UV–vis multicomponent analysis, fuzzy theory provides a model for the multicomponent spectrum based on standard spectra. Both the model and multicomponent spectra are weighted to minimize noise effects (fuzz). The degree of fit between the model and sample spectra generates a response surface which may be searched for the global optimum, *n*-dimensional co-ordinates of this optimum representing the concentrations of the *n*-components in the multicomponent mixture. The accuracy of the determination is limited by the noise (fuzz) on the spectra. The application of this method to the simultaneous determination of caffeine, propylphenazone and phenacetin appeared in the literature a considerable time ago [8]. Despite its apparent superiority over the least-squares estimate the method has attracted little interest, possibly due to the mathematical complexity of the technique.

There is increasing interest in the potential of NIR spectroscopy for *in situ* analysis of drug substances, excipients and drug products. The advantages of speed and simplicity make NIR an attractive technique for in-process control and finished product qualitative and quantitative analysis. Application of NIR reflectance spectroscopy to the determination of the active ingredient and water content of production batches of ceftazidime pentahydrate antibiotic powder samples has been reported [9]. It was found necessary to artificially broaden the range of values for the ceftazidime and water contents in order to obtain sufficiently accurate coefficients for the multiple linear regression. The authors found no problem with particle size/sample presentation effects and achieved a reliable assay for solid-state material. The advantages in eliminating sample preparation will ensure further refinement of NIR spectroscopy and wider application in the pharmaceutical industry, for example in the control of purity, including enantiomeric purity.

Chromatography

Contemporary pharmaceutical analysis is employing essentially all types of chromatographic separations such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), super-critical fluid chromatography (SFC) and capillary zone electrophoresis (CZE). The application of these techniques to the analysis of drugs, impurities and excipients in pharmaceutical preparations has been reviewed recently [10].

Thin-layer chromatography

Despite the long history of TLC in pharmaceutical analysis and its recent evolution into a heavily instrumented technique [11] it remains a method almost forgotten by chemometricians, with only method development receiving significant attention. An evaluation of discriminating power [12] or information content through principal components analysis [13] led to two groups proposing standardized TLC systems for pharmaceuticals. It is interesting to note that there is close agreement between these groups on two of the proposed systems but the wide divergence with the others suggests a more universal approach, applicable to individual needs, is required. Window diagrams, sequential simplex methods, statistical designs, the Prisma method, and mathematical modelling have all been advocated [14–18]. It remains to be seen whether future attention will be devoted to sample detection and data processing since, through the advent of scanning densitometers which are able to acquire UV–vis or fluorescence spectra, many of the peak deconvolution and peak purity techniques being employed in HPLC could be adapted to TLC.

High-performance liquid chromatography

For the pharmaceutical analyst, HPLC is very much a technique which complements TLC and it is likely that this situation will continue for many years to come. For the chemometrician, the combination of the wide utilization of HPLC, its high degree of automation and computer control, the information-rich detection systems that are available, and the versatility of method development processes made it an attractive area for research. Mobile phase optimization received much of the early attention, but more recently there has been an almost explosive growth in the application of expert systems to all areas of method development and validation. Chemometrics is also being extensively applied to post-separation data processing.

Mobile phase optimization. LC method development has been the subject of recent texts [19–21]. Systematic mobile phase optimization procedures are generally based on either sequential or simultaneous experimental designs. Of the sequential designs, the sequential simplex procedure is most often used [22, 23] since it is easily understood and requires no prior knowledge of the sample or chromatographic system. However, the sequential simplex procedure is restricted by its inability to distinguish reliably between local and global optima and thus, despite continuing advances to reduce the possibility of local optimum location [24] the simplex procedure is perhaps better suited to fine tuning when the global optimum region has been pre-defined. However, one area where the simplex procedure has been exploited recently to good effect is for the optimization of mobile phase composition for enantiomer separation [25]. Other applications of simplex methods were reviewed recently [22].

Simultaneous designs are largely based on regression methods as they aim to model one or more of the chromatographic parameters. Factorial or mixture designs are usually used and it is then necessary to model some aspect of the retention behaviour of the peaks in order to map the behaviour of the solutes under other conditions not explicitly tested by the initial, restricted set of experiments [26]. A full factorial central composite design was found [27] to be effective in defining separation conditions for metronidazole and tinidazole, and the same design was used in the development of a method for the separation of a complex mixture of monoamine neurotransmitters [28]. To meet the needs of the pharmaceutical analyst, not just the design of experiments needs consideration — it is important to consider the way the data will be analysed in order that maximum information is acquired. The previous two references describe graphical methods to represent the combined effects of variables on retention behaviour and enable a comparison between experimental and predicted results. It can be more important to be able to see how the *separation* between solutes changes as a function of the variables: this permits a rapid assessment of the robustness of a separation, a vital component of a fully validated assay which may require transfer to other laboratories, including those of regulatory agencies. This need has been emphasized in a review by Lankmayr *et al.* [29] who combined a factorial experimental design with a sequential strategy, including the use of chromatography simulation software, to determine the retention behaviour of five pharmaceutical compounds. The extent of the separation was then presented as a three-dimensional graph of a chromatographic

response function (CRF) which provides for an easy assessment of the ruggedness of the separation.

Similar information is available from two- or three-dimensional "window diagrams" and minimum resolution maps, both of which were used to develop the separation of 9-α-fluoroprednisolone from potential impurities [30]. The ultimate choice of the optimization method most appropriate to the problem at hand will always depend on the answer being sought. Recent reviews [31, 32] have compared mobile phase optimization methods.

Artificial intelligence in liquid chromatography. In the last 2 years there has been a rapid growth in the number of publications on the exploitation of artificial intelligence (AI) in pharmaceutical analysis, specifically the application of "expert systems" or "knowledge-based systems" for LC. Development of such systems has focused on LC because the knowledge domain of this technique is modular, e.g. sample preparation, method development and method validation, in which the basic rule sets are relatively well defined.

Moors and Massart [33] have investigated the feasibility of implementing knowledge into a small PC based expert systems shell KES (Knowledge Engineering System) that can advise on the solid-phase extraction of formulated drugs from aqueous solutions or plasma using a cyanopropyl sorbent. The system selects the most appropriate media to precondition and wash the CN-sorbent, then the eluent and selects a procedure for basic, acidic or neutral drugs with more than 10 carbon atoms and the degree of enrichment required. Although, further development of this system is required for the simultaneous extraction of the parent drug and metabolites in such matrices as urine, plasma and breast milk, it is a successful "first step" towards a knowledge-based system for bioanalysis.

Most knowledge engineers recognize the need to develop "rule-based" expert systems, for HPLC method development using a modular approach. In the case of the "rule-based" system the rules usually have been collated as a result of elicitation of both heuristic and algorithmic knowledge. They also acknowledge the apparent need to provide a recognizable acronym with which to label the software developed. Williams *et al.* [34] have developed a series of rule-based expert system programs written in the AI language Common LISP [35] collectively called ECAT (Expert Chromatographic Assistance Team), which comprises four modules connected to the Varian Inference Engine (VIS).

The Column and Mobile Phase module (CMP) is the key module in the system with approximately 300 rules which advises on the initial chromatographic conditions by building up a logic tree on the compound class and the physical and chemical characteristics. The Method Optimization module suggests a procedure for the optimization of the initial separation method produced by the CMP based on optimization gradient models [36, 37]. A third module (SPR) makes recommendations on the need for a guard column as a sample pretreatment to be incorporated into the method, or solid-phase extraction when the guard column fails to clean-up the sample adequately. The fourth component of ECAT is a database of chemical properties. This module at present contains 600 facts about the chemical properties which can be accessed by the other three modules. This adventurous project has modules for specific classes of compounds such as alkaloids or steroids.

A number of other workers in the field are at present developing analogous strategies in the exploitation of AI in HPLC method development, which also incorporate peak identification, on-line quantitation [38] and employ augmented planning networks (APN) as a knowledge representation model [39]. ESPRIT Project 1570 (European Strategic Program for the Research and Development in Information Technology) [40] has been instrumental in the development of the modular approach to Expert Systems for Chemical Analysis (ESCA) with particular emphasis on HPLC applied to pharmaceutical analysis. As a consequence, ESPRIT have evaluated a number of expert system tools that can potentially be employed in this particular application area [41]. One of the early ESCA products is LABEL [42] an expert system which advises on the most appropriate starting point for mobile phase composition and on the method of detection used [43] for the analysis of formulated drugs on a CN column based on the hydrophobicity, acid–base properties, number of carbon atoms and the UV or oxidative electrochemical activity of the molecule. The main application of such a system is the selection of an HPLC method to check the label claim of a formu-

lated drug substance: in the validation of the expert system rules the success rate was 82% for 44 formulations. One module DASH (Drug Analysis System in HPLC) [44], combines structural information with chromatographic knowledge to advise on "first guess" conditions for the separation and retention time optimization of basic drug candidates. For more than 50 compounds, the system provided 75% correct proposals [45]. Another compound-based system of interest to pharmaceutical analysis is CRIPES (Chromatographic Retention Index Prediction Expert System) [46]. Although, not currently containing drugs in its database CRIPES does provide an interface for the user to predict chromatographic retention from molecular structures. Darvas [47] has extended this approach to the development of an expert system HPLC–METABOL-EXPERT for the prediction of the possible metabolic transformation and the retention of the resulting metabolites of a basic drug.

There is no doubt that the trend of integrating optimization approaches will continue. One reported integrated system [48] advises on the selection of suitable optimization criteria (CRISE), guides systematic separation optimization used in conjunction with an automated interpretive optimization system, and then predicts the optimum hardware and operating conditions using a second expert system SOS (System-Optimization System).

Another such system embraces an introductory module which advises on whether ion-pairing or reversed-phase chromatography is more appropriate and the optimum mode of detection, an initial guess module, a formal module, and an adaptation module [49]. The formal optimization module is incorporated into the expert system which employs factorial design coupled to overlapping resolution mapping. Using this approach certain points are identified on the resulting contour map which have minimum resolution above R_s = 1.5 and acceptable k' values. The expert system then selects the conditions that produce the shortest run time. The adaptation module can advise on the experiment to further tailor the HPLC conditions based on 19 rules that encompass ways to change solvent strength and elution time. A high success rate was achieved for ion-pairing separations of 20 basic drugs and their 60 synthetic mixtures with this integrated approach.

A major challenge for the analyst is to demonstrate the validity of a given method. Validation of HPLC methods is complicated by the large numbers of variables which need accurate control. Another part of ESCA has been the development of expert system technology for method validation [50]. The challenge of method validation is large and this project required to be split into modules dealing with, for example, repeatability or ruggedness. The tool employed for the development of both packages is Goldworks [41] which can be readily interfaced to a Lotus 1-2-3 spreadsheet to perform on-line data manipulation required for method repeatability appraisal [51].

The ruggedness module initially identifies the factors that are likely to vary with the circumstances under which the method will be used, then efficiently employs Plackett–Burman fractional factorial designs to establish the impact of variations in experimental parameters on the quantitative performance of the method.

The resulting data are tested for precision to initially determine if they are repeatable or reproducible for quantitative analysis using peak areas and analyte concentrations, etc. They are then analysed to flag any possible chromatographic problems which can aid diagnostics for example by use of retention time–area ratios.

Expert systems are also being used to advise on algorithms for use with diode-array detector data in the assessment of peak homogeneity [52] using MircoProlog language developed specifically for a PC environment. This work indicated that the "knowledge-based system out-performs the human analysis in the recognition of homogeneous peaks".

Fault-finding and trouble shooting of separations and chromatographic hardware is another area in which the application of expert systems has been investigated for potential incorporation into a LIMS environment for automated on-line evaluation of chromatographic performance [53]. The rule-based system used is M.1 (Climflex Teknowledge) which is capable of analysing uncertainty by weighing up the evidence received and then establishing a priority for efficient trouble-shooting.

Finally, there are computer-based chromatographic simulators which can be regarded as modules of expert systems, the best known probably being DryLab [54] which requires 2–

4 initial isocratic runs, or 2 runs if the data are from gradient runs, to initiate the simulation process.

All the expert systems reviewed in this paper have been developed as a result of extensive knowledge elicitation to structure and formulate the expert's knowledge domain which can then be incorporated into the expert system. However, this process can sometimes be time consuming and may not readily capture the expert's intuitive process of solving problems. Rule induction expert systems are now being used more routinely in other areas to acquire rules and knowledge by the computer system learning as a result of analysing examples and extracting the probable rules which link the attribute to the classification in the examples analysed. One approach is based on the algorithm ID3 which develops a case classification tree. The system can also incorporate the use of a rule induction algorithm CX which is effective when there are uncertainties in the data or insufficient data for fully deterministic analysis.

Harrington and Voorhees [55] have developed a multivariate rule building expert system (MuRES) which combines the ID3 algorithm with linear discriminants. By generating rules from training sets of multivariate analytical data a linear combination of all variables is obtained which may result in the production of more efficient and effective classification trees. In future, such systems may offer considerable advantages for the capture of knowledge in chromatography.

At present there are still a number of limitations with the application of expert systems, e.g. refinement of the knowledge base can be difficult, if indeed the consequences of such knowledge refinements are acceptable. Many problems in pharmaceutical analysis are solved by only predominantly heuristic knowledge. Although rule-based expert systems can be applied successfully to the capture of this type of expertise, complimentary approaches in chemometrics such as genetic algorithms [56] and neural networks may be more appropriate as a learning system depending on the complexity of the analytical problem.

HPLC detection and signal processing. Chemometric methods have been applied for many years to detection in HPLC, with the focus being upon single and multi-channel UV detectors. Differentiation in the time domain [57] was used to enhance the resolution between tioconazole and process related impurities, thereby providing a more sensitive and robust method. Time domain digital differentiation processes are also useful for the removal of baseline disturbances, such as the drift encountered during gradient elution. By simple, repetitive re-integration of the derivative signal, the zero order chromatogram can be reconstructed without the baseline drift [1]. Such techniques should also be useful in the removal of matrix peaks, although modern data handling systems now provide the capability for the facile subtraction of "standard" or "blank" chromatograms to achieve this and the theory and application of this technique has been investigated recently [58, 59].

It is, however, the use of multi-channel (diode array) detectors which has spawned the majority of chemometric applications. Many optimization strategies require that peaks be treated during the process: achieving this is not straightforward. Wright *et al.* [60] showed that peaks could be tracked successfully using peak area data generated at multiple wavelengths. Of additional interest to the pharmaceutical analyst is the demonstration that it is possible to relate the total chromatographic peak integral to the UV absorbance of the sample derived from an independent, off-line measurement. The relationship derived provides a partial answer (i.e. $\pm 3\%$) to one of the often unanswered questions in HPLC — have all components been eluted? A comprehensive strategy for peak tracking in separation optimization has been developed by Strasters *et al.* [61]. Iterative Target Transformation Factor Analysis (ITTFA) is successful in tracking partially resolved, unknown components. This method has been incorporated into a commercially available methods development system [48], colloquially referred to as "diamond", which has been used successfully in the authors' laboratories for the development of methods to separate process related impurities, at $<1\%$, from the main band. No doubt such sophisticated systems will find increasing utility in pharmaceutical analysis, particularly as continuing software developments enhance their reliability, but many analysts still prefer the higher level of involvement in peak tracking that is available with more interactive systems (e.g. ICOS [62]).

The most readily visible application of chemometrics to diode array detection is in the

estimation of peak homogeneity. Most instrument manufacturers now build at least one method of accessing peak homogeneity into the software of their diode array detectors. The sensitivity of such methods varies according to the proportion of the co-eluted component, its separation and spectral similarity [63]. While some authors claim high sensitivity for their favoured method, none yet truly meets the needs of the pharmaceutical analyst monitoring drug substance purity or drug product stability, where sensitivities substantially less than 0.5% are required. Four different techniques, three involving peak suppression plus least-squares multicomponent analysis, were investigated for their efficiency for the accurate and precise determination of drugs in the low microgram range [64], with the least-squares method showing good potential. The authors have continued their studies by examining [65] absorbance ratios and principal components analysis in an automated peak-purity control procedure, using eight drugs of varying spectral similarities.

Examining the rationale for HPLC method to be used in pharmaceutical analysis, Szepesi *et al.* [66] employed absorbance ratios in combination with separations of mixtures subjected to stress conditions. Absorbance ratio methods tend to lack sensitivity but, by using multiple absorbance ratio correlation (MARC) [67], the sensitivity can be improved by up to an order to magnitude, enabling *ca* 1% of a co-eluting impurity of similar spectral characteristics to be determined. It is interesting to note that recent guidelines from the Canadian Bureau of Prescription Drugs [68] propose the use of absorbance ratioing for the verification of peak homogeneity for degraded samples.

Other forms of chromatography

Super-critical fluid chromatography (SFC) and capillary zone electrophoresis (CZE) are two newer chromatographic techniques which will complement HPLC in pharmaceutical analysis [69]. Of the three methods, SFC possesses the narrowest range of applications, with retention showing some correlation with HPLC. CZE, on the other hand, shows no obvious correlation with HPLC [69], suggesting them to be orthogonal systems with respect to their information content. SFC separations have been optimized using the simplex algorithm [70] and by an interpretive procedure [71].

Finally, Bachman and Stewart [72] have been studying the use of chemometric methods in the optimization of electrochemical response obtained by photochemical reaction detection. They have reported recently the first multivariate approach to detection optimization, using factorial designs as applied to the determination of antihypertensive drugs in dosage forms and biological fluids.

Conclusion

By far the widest applications of chemometrics in pharmaceutical analysis remain directed at chromatography, followed by spectroscopy. The increasing emphasis is to move towards more "intelligent" systems. Such developments have both a price and a consequence. The price is one of increasing dependence upon "state-of-the-art" computer controlled instrumentation, and the need for scientists of appropriate training and expertise to develop and validate applications. The consequences are, hopefully, more efficient analyses providing far greater economics and dependability (i.e. safety and efficacy) of drug substances and products. Through all this is seen an explosion of information. Chemometrics can help here too, in its efficient capture and organization, in its facile transmission and review by regulatory authorities, and by assisting in its utilization (e.g. through knowledge-based expert systems) in the QC environment [73].

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