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VOLUME 19 NUMBER 12 1996

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

July 1996

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Identification Statement. *Journal of Liquid Chromatography & Related Technologies* (ISSN: 1082-6076) is published semimonthly except monthly in May, August, October, and December for the institutional rate of $1,595.00 and the individual rate of $797.50 by Marcel Dekker, Inc., P.O. Box 5005, Monticello, NY 12701-5185. Second Class postage paid at Monticello, NY. POSTMASTER: Send address changes to *Journal of Liquid Chromatography & Related Technologies*, P.O. Box 5005, Monticello, NY 12701-5185.

Volume	Issues	Institutional Rate	Individual Professionals' and Student Rate	Foreign Postage		
				Surface	Airmail to Europe	Airmail to Asia
19	20	$1,595.00	$797.50	$70.00	$110.00	$130.00

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CODEN: JLCTFC 19(12) i–iv, 1873–2038 (1996)
ISSN: 1082-6076

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Contributions to this journal are published free of charge.

Effective with Volume 6, Number 11, this journal is printed on acid-free paper.

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1873–1891 (1996)

CHROMATOGRAPHIC METHOD VALIDATION: A REVIEW OF CURRENT PRACTICES AND PROCEDURES. III. RUGGEDNESS, RE-VALIDATION AND SYSTEM SUITABILITY

Dennis R. Jenke

Baxter Healthcare Corporation
William B. Graham Science Center
Round Lake, IL 60073

ABSTRACT

Validation of analytical methodologies is an important aspect of their development/utilization and is widely required in support of industrial product development and registration. In this manuscript, ruggedness as a validation parameter is considered in terms of its definition, appropriate evaluation procedures and acceptance criteria. Additionally, the re-validation of analytical methods is discussed, strategies for the effective development and utilization of system suitability tests are described and the term "stability indiating" is defined.

INTRODUCTION

Chromatographic methods are used for the quantitative and qualitative characterization of environmental and pharmaceutical samples. The object of the characterization is to generate a reliable, accurate and interpretable set of information describing the sample. To ensure that an analytical procedure fulfills this objective, it undergoes an evaluation loosely termed validation. In previous parts of this series,[1,2] primary validation parameters (e.g., accuracy, precision, specificity, linearity and sensitivity) were identified and discussed in

terms of their definition, scope, evaluation procedures and acceptance criteria. In this manuscript, the series is concluded with a consideration of ruggedness, re-validation and system suitability.

RUGGEDNESS

Definition

It is generally expected that an analytical method will perform in an acceptable manner each time it is used. A method which is difficult to implement is highly undesirable from the practical perspective of efficient resource utilization and is generally suspect in terms of the quality of the data generated. The ability to routinely implement an assay reflects its inherent ruggedness. While a consideration of method ruggedness is a necessary part of any method's validation, it's a critical issue for compendial methods because of their widespread use in many different laboratories.[3]

Ruggedness establishes a method's ability to perform effectively in the face of variations which can reasonably be expected to occur whenever the method is implemented. More specifically, ruggedness is the reproducibility of test results obtained by the analysis of samples under a variety of normal test conditions such as different laboratories, analysts, instruments, reagent lots, elapsed assay times, temperatures, etcetera.[3-7] Thus, ruggedness addresses ***unintentional*** variation in the method introduced by its application, at different times by different people at different locations using different instrumentation and materials. Ruggedness measures the extent to which a method is sensitive to small changes in procedures and circumstances.[8] A rugged method will be able to withstand minor operating or performance changes[9] and has built in buffers against typical procedural abuses, such as, differences in care, technique, equipment and conditions.[10]

Procedures

Clearly, ruggedness is assessed by implementing the analytical method under different operational conditions. The ruggedness test should be performed at several values of each operational parameter which affects method performance.[3] For chromatographic assays, these parameters might include mobile phase composition and flow rate, column vendor, column condition, detection wavelength, sample and standard preparation procedures and operating temperature. For a reverse phase HPLC method using an ion pairing reagent, for example, the following conditions can be evaluated for their effect on capacity factor(s) or resolution of a critical pair of analytes:[11]

* Mobile phase composition (pH, buffer concentration, ion pairing reagent concentration, percent organic phase),
* column temperature,
* injection volume,
* gradient dwell time, and
* column lots or column manufacturers.

The ruggedness test should be performed by analyzing aliquots from homogeneous sample lots using operational and environmental conditions that differ but are still within the method's specified operating range.[4,9,12] The ruggedness evaluation should be performed on a sample which has been previously characterized (especially in terms of its stability) by an experienced analyst[13] and should include any precision-related tests and requirements contained in the procedure's protocol or specification.[14]

In order to assess the magnitude of operator-related ruggedness, it has been suggested that four analysts perform one assay per day for three days.[5] The utilization of statistically designed experiments (e.g., Plackett-Burman, nested ANOVA, factorial plans) to establish the ruggedness of an assay is strongly recommended.[15,18]

A common source of performance variation in chromatographic methods is the separation column. Performance variation is introduced into the method by the age and care of the column, inherent column non-reproducibility resulting from production variations within a manufacturer's process (batch of stationary phase, packing procedure) and variation in selectivity and performance between columns of similar generic type supplied from different vendors in different configurations.

In order to assess column ruggedness, it is recommended that the specificity of at least three columns, each one from a different batche produced by the recommended column manufacturer and at least one column from a different manufacturer be checked.[3,10,14]

Acceptance Criteria

The quantitative measure of a method's ruggedness is the precision behavior it exhibits over the course of the various operational scenarios examined during the validation exercise. To determine the method's ruggedness, method reproducibility obtained throughout the changes tested, should be compared to the precision of the assay under normal conditions;[4] the reproducibility thus obtained should not be significantly different from the method's intermediate precision obtained under normal operating conditions. Generally, a rugged method's reproducibility is 2 to 3 times greater than the

method's repeatability [inherent method precision under "normal" controlled operating conditions].[17] For ruggedness determinations utilizing a factorial design, a ratio of the variances associated with ruggedness and reproducibility of greater than 1.5 is strongly indicative of one or more factors that adversely affect the method's performance.[18] For an evaluation of column to column ruggedness, it is required, in addition to a precision comparison, that the method pass the specificity test criteria on all columns tested.[14]

Related Considerations

Application of chromatographic procedures requires the use of liquid samples, standard and related analytical reagents. In most routine applications, solutions are not used immediately after preparation but may be stored under specified conditions prior to use. Verifying solution stability is an important aspect of method validation; specifically, a valid method is one for which all related and analytical solutions are stable over the period typically required for their utilization/analysis. To address stability, the analytical solutions should be prepared, assayed, allowed to stand (in accordance with the method's protocol or specification) for a length of time equal to the anticipated maximum analysis time and then re-assayed.[14,19] It has been suggested that, for analytical scenarios involving overnight runs, four sample solutions over the working concentration range should be analyzed repetitively over the course of at least sixteen hours.[20] In such evaluations, the analytical solution is stable if all concentration values obtained before and after storage agree to within three times the system precision.[14,19] Additionally, no new peaks should appear in, nor should existing peaks be lost, from the chromatograms of the first and last sample injection.[14]

While ruggedness is related to unintentional variation in a method due to its use in varying analytical situations, method robustness is a measure of a procedure's capacity to remain unaffected by small but ***deliberate*** variations in method parameters and thus is a measure of the procedure's reliability during normal usage.[4,13,21,22] Although time consuming to perform, thorough robustness studies will help avoid unexpected results in subsequent applications of the method. Thus, the robustness evaluation should serve as a prelude to assay transfer.[13] While data for robustness is not usually submitted in regulatory product applications, a robustness evaluation is recommended.[22]

It has been suggested that in order to determine robustness, a method's critical operational variables should be identified by breaking the testing process up into unit operations and then assessing the potential variability of each such operation.[13] Unit operations might include:

1. Analytical solution preparation: amount of material used, volumes of solvent used, dissolution times and conditions, solvent used.
2. Variation in the tested product (inhomogeneity, aging).
3. Instrumental analysis: detection wavelength, mobile phase composition and flow rate, column use history.

The intent of the robustness evaluation is to quantify the amount of method variation introduced by changing an operational variable by a known amount. Clearly, a robust method is one which is operationally immune to commonly encountered but relatively minor variations in its critical operating parameters.

METHOD RE-VALIDATION

If an analytical method exhibits any significant longevity, it invariably undergoes some change in procedure or implementation. It is possible that method performance, and thus the validity of the data generated by the method, could be adversely impacted by such changes. Re-validation, which may be required in such situations, is the reassessment of a validated analytical method in response to a change in some aspect of the method.

Issues associated with re-validation are two-fold: 1) how big of a change triggers a re-validation and 2) how extensive should the re-validation be? Considering the former, utilization of the most conservative approach minimizes the likelihood that even the most apparently innocuous change could produce a significant change in performance. Specifically, the investigator must avoid assumptions regarding the definition of a "major" change[13] and assume that any modification of the analytical method would require re-validation.[23] In essence, validation should be ongoing in the form of re-validation with method changes.[14] For chromatographic methods, significant changes could include:

* Changes in the product for which the method was validated,
* Use of the assay for a product different from that for which it was validated,
* Instrument changes,
* Reagent changes (type or vendor),
* Procedural changes,
* Personnel changes, and
* Technological changes (e.g., developments in column and/or instrumentation technology).

With regard to the extent of the re-validation exercise, it is clear that the greater the magnitude of the method change, the greater the need for and scope of the re-validation.[24] The decision regarding which parameters require re-

Table 1

Method Changes and Re-Validation Tests Required

Method Characteristics Changed	Performance Parameters to Re-validate
Instrument Changes	Linearity (working range), LOD, LOQ, system precision
Product Changes	Selectivity, accuracy, precision
Sample Preparation Procedure (same solvent, same concentration range)	Accuracy, recovery, precision, ruggedness
Sample Preparation Procedure (different solvent, different concentration range)	Complete-reassesment of all previously used validation parameters
Analyst Changes	Qualification testing (perform re-tests, side by side collaborative studies)
Chromatographic change (e.g., column, mobile phase)	Selectivity, linearity, LOD/LOQ, system precision

LOD = Limit of Detection, LOQ = Limit of Quantitation.
From reference 13.

validation should be based on a logical consideration of the specific validation parameters which are likely to be affected by the change.[16] Minimally, however, re-validation of chromatographic methods might include an assessment of accuracy and the absence of interference[4] or the running of a standard curve with new quality control samples to show that the response relationships and general characteristics of the "new" method are similar to the previous validation results.[23] For bio-analytical methods, precision, accuracy and limit of quantitation are considered to be the minimum re-validation tests.[24] More specific recommendations for which method parameters should be re-validated in response to specific types of procedural changes are summarized in Tables 1 and 2.

SYSTEM SUITABILITY

Role

To obtain a good and acceptable analytical result, two requirements must be met; (1) the method has to be adequate and (2) the execution has to be adequate.[25] In its broadest sense, method validation addresses the former issue

Table 2

Additional Guidelines for Re-validation

Method Characteristics Changed	Performance Parameters to Re-Validate
Extraction solvent, buffer, back extraction matrix or injection solvent	Linearity, recovery, LOQ, intra-batch preciison and accuracy in process solution stability. Additionally, if injection solvent is changed, processed sample stability should be checked but recovery or in-process stability checks are not necessary.
Chromatographic conditions [column, mobile phase composition, detector type or monitoring condition (e.g. wavelength) change].	Linearity, selectivity, intra-batch precision and accuracy (recovery not necessary).
Extending the upper end or reducing the lower end of the calibration curve range.	Linearity, LOQ (if reduced), intra-batch precision and accuracy at revised upper or lower levels.
Internal standard	Selectivity, intra-batch precision and accuracy, recovery.

From reference 16.

but leaves the latter essentially unresolved. While the most rigorous verification of adequate execution would be re-validation at each use, such an approach suffers from serious practical shortcomings involving resource constraints. System suitability tests (SST) have been adopted by chromatographers to describe the process by which the execution of an analytical process is evaluated. SST typically represents a sub-set of the method validation procedures and obviates the need for a more rigorous re-validation[10] by serving as a surrogate for the more involved validation process.

SST tests, introduced by FDA chemists in the early 1970's,[26] were originally intended to prevent the known variability of chromatographic components from adversely affecting official methods. Even today, the USP monograph on Chromatography[27] indicates that the SST "are used to verify that the resolution and repeatability of the chromatographic system are adequate..." and that resolution, tailing factor and precision are the primary SST parameters. As SST procedures became a more common part of the method development/utilization process, their traditional role has been enumerated by

numerous authors. Such descriptions suggest that the role of SST testing is to:

* Confirm the method's continuing suitability for use.[28,29]
* Ensure that the method is performing properly, satisfactorily or as intended.[8,10,24,30]
* Establish that the system meets criteria of historic norms, accepted operational standards, or performance requirements.[3,5,10]
* Provide the analyst with an early warning that an analytical process is likely to be out of control.[31]

Historically then, the SST has been implemented as a time of use procedure, whose sole purpose was to document acceptable system operation by comparing observed performance versus previously established guidelines. While it served an important role in such applications, the impact of the SST was both passive and reactive. Although the test identified a sub-optimal system, it provided no clue as to how to improve performance. Additionally, the performance of the SST was most commonly viewed as a one time event, with little or no effort made to interpret trends in SST data as a means of proactively recognizing decaying system performance. More recent manuscripts, have suggested more active roles for SST evaluations including:

* A correctly used SST should verify that the analysis has been performed consistently over time.[29]
* A SST should indicate which component in or step of an analytical procedure should be replaced or modified.[29]
* The data should be useful as a means of directing a non-compliance towards a compliance.[30]
* The SST must indicate what the analyst should do in the event of a test failure.[11]

Thus, in its evolving role, the SST serves not only as an indicator of adequate performance but also provides diagnostic information related to the source of the problem and prescriptive information related to the correction of the problem. Through the use of control charting, the SST database provides a picture of the system's historical capabilities and allows for the development of statistically based performance criteria. In this expanded role, the SST is a vital tool for the routine quality control of chromatographic assays.[30]

Previously, an effective validation plan was defined as one for which the user knows which performance parameters to assess (scope), how parameter evaluation is performed (procedure) and the appropriate acceptance criteria are.[2] A similar definition is appropriate for an effective SST. The following discussion considers these aspects of an SST evaluation in greater detail.

Table 3

ParametersWhich Should be Contained in a System Suitability Evaluation (Based on a Survey of Published Methods on the LC Analysis of Drug Substances and Dosage Forms)

Evaluation Parameter	Frequency of Citation (#)
Resolution	23
Precision of Standards	17
Standard Linearity	7
Tailing Factor	6
Theoretical Plates	5
Retention Time	2
Precision of Impurities	1
Capacity Factor	1
Peak Asymmetry	1

(#) Number of citations which mentioned this specific SST parameter. Of the 84 total references city in this manuscript, 28 provided system suitability test guidelines.
From reference 34.

Scope

Critical issues associated with performing a system suitability test include the identification of which performance characteristics need to be monitored and how frequently the test must be performed. The overriding issue here is efficiency; it is desired that the test provide the maximum measure of system performance with a minimum expenditure of time and effort.[29] The design of the system suitability test should balance the time to perform the test versus the risk of chromatographic failure during the run (and the resulting non-availablilty of the analytical data).[29] Historically, this balance has been heavily weighted against rigorous SST testing, which is often viewec as a formality to be overcome. However, evaluation of a system with a properly written SST may actually save more analytical time than is taken to perform the test by eliminating retesting.[14]

Ultimately, the amount of testing performed will depend on the purpose and nature of the test method.[22] While an SST should be considered for each parameter which was checked during method validatior.,[28] the implemented test procedure should incorporate only those key parameters that are crucial to the success of the method, as defined by its specific analytical objectives.[29] For example, while an SST for sensitivity might be quite applicable in an impurity

assay (where the ability to detect the impurity is important), such a test might have little application in situations wherein the intent of the assay is to accurately quantitate a formulation component present in the sample in large quantities.

Since this manuscript is limited to a consideration of chromatographic methods, appropriate SST parameters must reflect problems associated with the implementation specifically of chromatographic procedures. Problems a thorough system suitability test should surface include;[32]

* flow irregularity,
* injection irreproducibility,
* system plumbing problems,
* detector mis-alinement/malfunction,
* column malfunction, and
* mis-preparation of analytical solutions (mobile phase, sample diluent, derivatization reagent, standards, samples).

Numerous authors have outlined parameters which should be examined in a rigorous SST, e.g. references 2, 7-12, 22, 27, 28, 32 and 33. Two parameters mentioned in every manuscript examined for this review were resolution and repeatability (e.g., system precision). The universal use of these parameters is understandable since they touch on two important properties of the chromatographic assay, specificity and precision. A measure of peak shape (e.g., tailing factor, peak asymmetry) was also frequently cited as a necessary component of a rigorous suitability assessment. Assessment parameters which were less frequently noted included capacity factor (ratio), a measure of sensitivity (LOD or LOQ), linearity, column efficiency (plate count) and the analysis of controls. The use of multiple injections of a standard, made throughout the run to assess response stability, was also suggested.[11]

These observations are reinforced by two recently published surveys. In 1990, T. D. Wilson published the results of a survey of literature methods on the LC analysis of drug substances and dosage forms.[34] Of 84 references cited, 28 made specific mention of system suitability parameters. As shown in Table 3, the frequency with which specific SST parameters were mentioned mimics the general trend noted previously. Additionally, in 1994, G. S. Clarke surveyed most major pharmaceutical companies with research laboratories in the UK with respect to their method validation and system suitability procedures.[35] Data summarizing the frequency with which specific SST parameters were used are contained in Table 4. Parameters which were used by a majority of the companies included precision, selectivity (resolution) and chromatographic performance (e.g., resolution, efficiency) while accuracy, linearity, selectivity, ruggedness, solution stability and sensitivity (signal to noise ratio) were used less commonly.

Table 4

Parameters Which Sould be Contained in a System Suitability Evaluation (Based on a Survey of Practices Used by Major Research-Based Pharmaceutical Companies With Laboratories in the UK)

Parameter	Company Responses (*)	
	In Common Use	Not in Common Use
Selectivity (resolution)	20	0
	14	4
Chromatographic parameters (capacity factor, plate count, tailing factor)	11	9
Limit of Detection (LOD)	9	11
Accuracy	4	16
Linearity	3	15
Signal to Noise ration	1	19
Selectivity (peak homogeneity)	1	19
Solution Stability	1	9

(*) The total number of companies survey was 20.
From reference 35.

Wahlich and Carr[28] advocate the use of SST parameters which reflect each parameter which was considered as part of the method's validation process. These parameters, linked to typical validation parameters and contrasted to the more conventionally recommended SST parameters, are summarized in Table 5.

In reviewing the literature related to SST parameters, this author was struck by several points. Firstly, it is somewhat unusual, in this author's opinion, that some direct measure of accuracy was so infrequently cited as a necessary SST parameter. This is striking since accuracy is one of the most universally applied method validation parameters, Except in bioanalytical procedures, wherein analyzing QC samples is the most popular method for monitoring assay performance,[24] the direct assessment of method accuracy is rarely mentioned as a necessary SST parameter.

Secondly, there exists some discordance in terms of which of the chromatographic performance parameters are most useful. For example, several authors suggest that "it is questionable whether in absolute terms either tailing or column efficiency add anything to the suitability for use of a method".[28] They suggest this is true since little attempt is usually made to determine whether failure to comply with criteria for these parameters means that the method is any

Table 5

A Suggested Link Between Method Validation Parameters and System Suitability Tests

Method Validation Parameter	Traditional SST	Recommended SST
Ruggedness/robustness	None	Check on critical method parameters
Accuracy	None	Control sample, re-extraction or mass balance
Precision	RSD of replicate injections	RSD of replicate injections: RSD of replicate sample preparations
Selectivity	Resolution check	Resolution check (using impure standards or samples of the impurities)
Stability of the measurement system	None	Comparison of standards at the start and end of run
Linearity	None	Use of standard at different concentrations
Signal to Noise (LOD/ LOQ)	None	Calculation of H/s_B rato (*)
General Acceptability	None	Chromatogram compared to reference chromatogram
None	Tailing factor/peak asymmetry	None
None		None
None	Column efficiency/plate count	None

(*) H = peak height of a specified standard; s_B = standard deviation of the baseline.
From reference 28.

less valid. Additionally, neither peak tailing or efficiency has any direct link to a primary validation parameter. Considering tailing, it has been observed that as peak asymmetry increases, accuracy[22] and precision[27,29] suffer. Thus peak tailing acts a surrogate SST for accuracy and precision. Since precision is a routinely utilized SST parameter, and the SST assessment of accuracy is becoming more common, the usefullness of the peak shape SST is questionable.

A similar situation exists for efficiency. Efficiency is utilized as an SST to indirectly assess method specificity; that is, efficiency seeks to ensure that the column possess the ability to separate the analytical peak(s) of interest from all

possible interfering responses. While most investigators acknowledge that an assurance of specificity is an important SST component, it is frequently noted that efficiency (theoretical plates) is inferior to resolution[14] as a measure of specificity. Resolution may be considered to be a more powerful tool for testing chromatographic performance since it addresses efficiency (N), selectivity (alpha) and capacity (k') via the expression:[30]

$$R = 1/4 \times (\text{alpha} - 1) \times N^{0.5} \times [k'/(1+k')]$$

Procedures

The first procedural aspect to be considered is the frequency with which an SST should be performed. Two timeframes are pertinent; within run repetition of SST testing and between run testing. Considering within run replication of SST testing, the current USP emphasis is to perform all system suitability injections prior to the analysis of actual samples.[27] However, such an approach can lead to erroneous results since it establishes only that the system performed within expectations at the beginning of the run and does not demonstrate that such performance was maintained throughout the run.[36] In general, intervals between tests should be shorter than the observed time in which the system drifts outside of acceptable levels.[37] In most cases, this means that the SST is performed at the beginning and end of the run. Such testing can take the form of a precision evaluation to ensure that the nature of the analytical response has not changed over time, or may involve nothing more complicated than a visual comparison of chromatograms generated at the beginning and end of the run, from the same sample.[28] More rigorously, it has been proposed that the appropriate frequency for the SST test, is to run one QC control per every ten samples or, for short runs, two QC controls minimum,[24] while tests for bias and/or response stability should include the repetitive analysis of a single solution throughout the run.[10,11,33] Additionally, an SST evaluation is performed each time an instrument malfunction has been identified during the course of a run.[27]

The decision of how frequently an SST is performed between analytical runs should be determined by experience and based on need, type of test and equipment and previous performance of the equipment.[37] Minimally, the SST should be performed in full each time the system is assembled for the assay. However, if the system is in continuous use for the same analysis, then it may be sufficient to perform an abbreviated SST check each day.[14]

Considering other procedural aspects of system suitability testing, several authors provide somewhat more quantitative guidelines on how the SST is to be performed. When utilizing QC samples to assess accuracy, it is suggested that duplicate injections be made of QC standards at three concentrations [below,

Table 6

Recommended System Suitability Test Acceptance Criteria

Parameter	Assay Type	Acceptance Criterion	
		Hsu and Chien(*)	CDER (#)
Capacity Factor			
	General	2 to 8	> 2
	Trace	1 - 3	N/A
	Stability Indicating	> 4	N/A
Selectivity	General	1.05 to 2.0	N/A
Resolution	General	>2.0	>2.0
	Quantitative Analysis	>1.5	N/A
	Biologicals	>1.2	N/A
Plate Count (N)	General	(a)	>2000
Precision	General	% RSD ≤ 1.5%	% RSD ≤ 1.0% (b)
	Biologicals	% RSD ≤ 5%	N/A
	Trace	% RSD, 5 to 15%	N/A
Tailing Factor	General	1.5 to 2.0	≤ 2.0

Notes: (a) = no criterion given, however, the analyst should look for decreases in this number as a sign of degrading system performance.
(b) = for 5 replicate injections.
N/A = no specific guidelines given for this situation.
(*) reference 40; (#) reference 22.

within (midpoint) and above] around the expected range.[23,28] To assess system precision, samples at both ends of the calibration curve should be injected at least five times, with six injections being required if the acceptance criterion is a %RSD greater than 2.0%.[14,27,29]

Acceptance Criteria

The acceptance criteria established for the SST evaluation must balance the need to insure adequate performance with the practical reality of performing chemical analyses. Thus, the criteria must be sufficiently tight that data quality is assured but not so restrictive that perfectly acceptable systems cannot readily pass all criteria. It is crucial that the acceptance criteria are designed to reflect method variances which affect the quality of the data generated.[11] To be useful to the analyst, the criteria should reflect minimum, as opposed to typical, performance.[30]

In general, setting the acceptance criteria involves an assessment of the chromatographic conditions to which the method is most sensitive and then using existing performance data (obtained perhaps during method development-validation), to help establish the criteria.[30] Typically, data obtained from ruggedness testing, can help define system suitability criteria.[39] It has been proposed[30] that the following three step process be used to develop meaningful system suitability criteria:

(1) Determine the sensitivity of the method to changes in chromatographic conditions.
(2) Identify suitable performance parameters that can monitor system functionality and determine their minimum or maximum acceptable value.
(3) Validate these criteria for each formulation, product or sample that is assayed by the method.

Specific recommendations for SST acceptance criteria include:

* For the repetitive injection of response stability samples, the %RSD of the repetitive injections should be ≤ 120% of the system precision.[33]
* Duplicate injections of a standard injected periodically throughout an assay should agree to within 0.5% of their average.[10]
* The %RSD of a series of standard injections interspersed throughout the run should have a %RSD ≤ 1%. Failure to comply with this criterion may be overcome by using standard bracketing to divide the run into "compliant" portions [i.e., portions which meet the criterion].[28]
* In using QC samples, the results are acceptable if they are within 10% of the known value.[38]
* For QC protocols involving the duplicate analysis of samples prepared at three concentrations (e.g., biological samples), 4 of the 6 QC values must be within 20% of expected, while those outside this range cannot be of the same concentration.[23]

More detailed acceptance criteria are provided for the common SST parameters by Hsu and Chien[40] and the Center for Drug Evaluation and Research [FDA][22] and are summarized in Table 6.

SST Failures

If a system fails an SST and the procedure specification or protocol describes the analytical procedure in great detail, the analyst is faced with the dilemma of what to do next. Fortunately, it is well recognized in the pharmacopeial literature, that the specification of definitive parameters in a

monograph (procedure) does not preclude the use of other suitable operating conditions and, thus, that adjustments of operating conditions to meet the system suitability requirements may be necessary and appropriate.[3,27] However, once the conditions have been adjusted, it is not adequate to test the new system only for that SST which was previously failed. Utilization of the adjusted system is predicated on the assumption that its is capable of meeting all SST requirements.

STABILITY INDICATING ASSAYS

Assays suitable for the determination of the stability and shelf life of pharmaceutical formulations and products share expected performance criteria which are somewhat more rigorous than those necessary for assays used in other applications. A stability indicating assay must be able to determine small changes in the concentration of the analyte of interest and exhibit no interference from other sample components (e.g., degradation products).[41] Special demands placed on stability indicating assays include:[33]

* The method should be able to accurately follow the decrease in active content during the period of the stability investigation,
* The desired resolutions between peaks are set higher (than in most other applications) in order to identify and quantitate degradation products,
* Reproducibility (day to day precision) must be better than 1% RSD in order that small decreases in active ingredients can be measured, and
* the peaks of the primary and secondary degradations products must be separated from one another, the active ingredient and other formulation impurities.

Stability indicating assays, typically quantitate analytes which include one or two major componentes and several impurities (<0.5%). These assays have resolution (between multiple peaks), accuracy, reproducibility and sensitivity as primary validation and system suitability parameters.[7] Thus, one can expect the acceptance criteria for these assays to be more stringent than for those assays used in other pharmaceutical situations.

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Received December 4, 1995
Accepted December 11, 1995
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J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1893–1908 (1996)

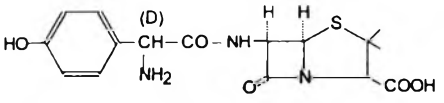
EVALUATION OF LC METHODS FOR THE SEPARATION OF AMOXICILLIN AND ITS RELATED SUBSTANCES

Zhu Yongxin[1], E. Roets[1], M. L. Moreno[2], E. Porqueras[2], J. Hoogmartens[1]

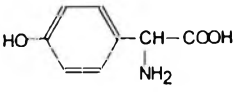
[1] Katholieke Universiteit Leuven
Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen
Faculteit Farmaceutische Wetenschappen
Van Evenstraat 4
3000 Leuven (Belgium)
[2] Centro National De Farmacobiologia
Instituto De Salud Carlos III
28220 Majadahonda, Madrid (Spain)

ABSTRACT

Five isocratic liquid chromatography (LC) methods have been examined for the separation of amoxicillin and its related substances on C_{18} or C_8 columns. The United States Pharmacopeia (USP) assay method gave better selectivity. Similar selectivity was obtained not only on C_{18} columns but also on C_8 and poly(styrene-divinylbenzene) columns. The good selectivity was confirmed by a second laboratory. A resolution test using cefadroxil was developed for the method performance. Based on the USP method, a gradient LC method was developed for the analysis of related substances in amoxicillin. This method has been proposed for the assay and purity control in the amoxicillin monographs of the European Pharmacopoeia and will be further examined in a collaborative study.



1. amoxicillin



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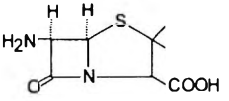
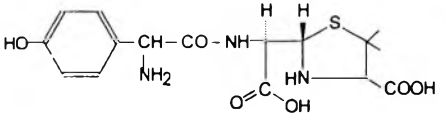


3. 6-APA



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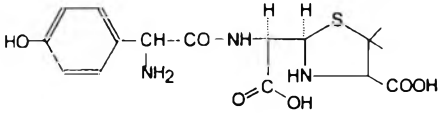
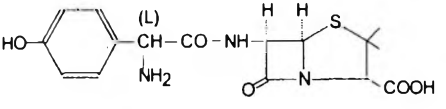
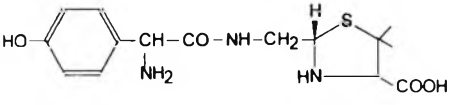


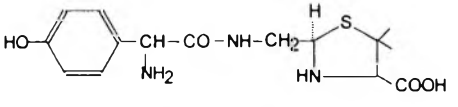
5. amoxicilloic acid (5R, 6R)



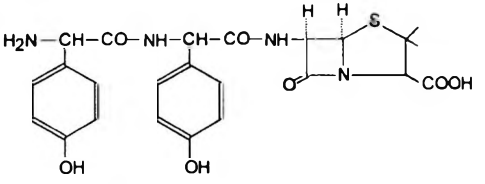
6. L-amoxicillin



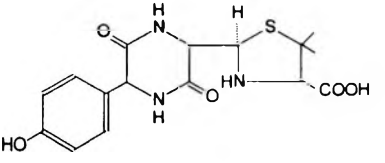
7. amoxiiloic acid (5S)



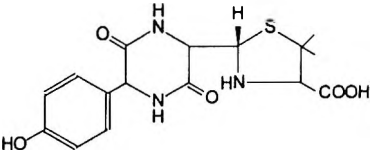
8. amoxiiloic acid (5R)



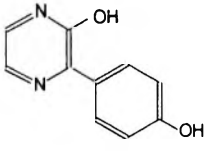
9. 4-hydroxyphenylglycylamoxicillin



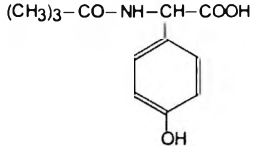
10. amoxicillin (5R) piperazine-2',5'-dione



11. amoxicillin (5S) piperazine-2',5'-dione

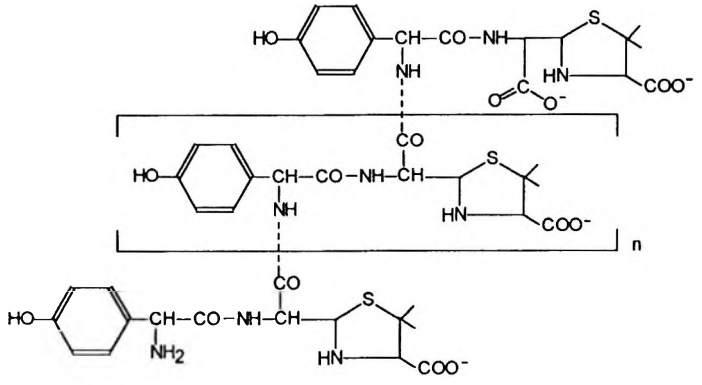


12. 2-hydroxy-3-(4-hydroxy)phenylpyrazine

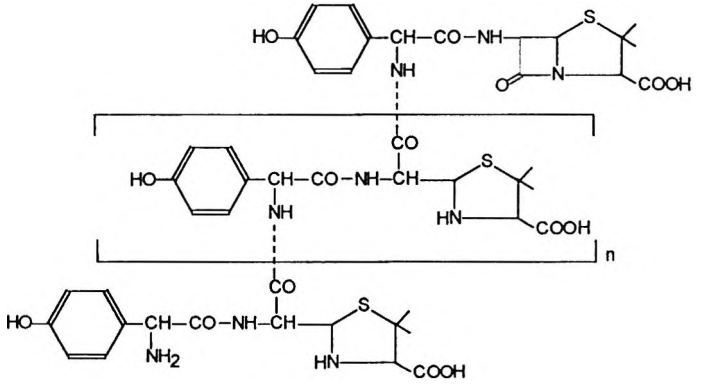


13. N-pivaloyl-4-hydroxyphenylglycine

Figure 1. Structures of amoxicillin and its related substances.



14. dimeroates (n = 0)
trimeroates (n = 1)



15. dimer (n = 0)
trimer (n = 1)

Figure 1 (cont.). Structures of amoxicillin and its related substances.

INTRODUCTION

Amoxicillin is a semi-synthetic penicillin with activity against both gram-positive and gram-negative bacteria. It is available as injectable, capsule, and oral suspension. Amoxicillin may contain precursors, side products from the semi-synthesis and various degradation products including oligomers. Fig. 1

shows the structures of amoxicillin and a number of related substances, which were available. Some of the related substances were kindly donated by manufacturers, but most had to be prepared in the laboratory.

LC methods are quite often used for the analysis of amoxicillin. Some papers on amoxicillin describe the determination of amoxicillin in biological samples.[1-2] These methods highlight the separation of the antibiotic from the background of biological materials.

Some papers discuss the separation of amoxicillin from other penicillins or other drugs.[3-8] Some papers deal with the measurement of amoxicillin by using special detection techniques.[9-11]

A number of papers report the determination of amoxicillin in pharmaceuticals.[12-14] LC is widely used by manufacturers for assay of amoxicillin and is also prescribed by the USP.[15] However, the separation of amoxicillin and its related substances and the reproducibility of the selectivity on different columns have not been sufficiently discussed. This was mainly due to non availability of some related substances.

In the present study, the selectivity of five isocratic LC methods for assay has been examined. Two were taken from literature,[12,14] one from the USP[15] and the other two were made available by manufacturers of amoxicillin. Table 1 shows the LC conditions of the five methods. All use C_{18} stationary phases except method III, using C_8. The prescribed conditions were slightly adapted in our study, as shown.

The aim of the present study is to examine whether an existing assay method was sufficiently selective and whether it could be adapted in order to be suitable as a related substances test.

EXPERIMENTAL

Samples

Amoxicillin trihydrate and amoxicillin sodium are commercially available. Related substances originate from the semi-synthesis and from degradation. The structures of the available related substances are shown in Fig.1. D-4-Hydroxyphenylglycine (**2**) and 6-aminopenicillanic acid (6-APA, **3**) are the basic constituents of amoxicillin and are commercially available. L-amoxicillin (**6**), 4-hydroxyphenylglycylamoxicillin (**9**) and N-pivaloyl-4-hydroxyphenyl-glycine (**13**) can arise from the semi-synthesis of amoxicillin. Related

Table 1

LC Conditions for Five Isocratic LC Methods

Meth.	Source	Mobile Phase Prescribed	Column Temp. Prescribed	Flow Rate (mL/min) Prescribed	Detection* UV nm Prescribed	Column Temp. Used	Flow Rate (mL/min) Used
I	Mfr. 1	0.02 M phosphate buffer pH 5.0-CH_3OH (93:7)	40°C	1.5	230	40°C	1
II	Mfr. 2	0.1 M phosphate buffer pH 4.5-CH_3OH (95:5)	Ambient	---	230	30°C	1
III	LeBelle	0.05 M phosphate buffer pH 5.0-CH_3OH (94:6)	30°C	1	254	30°C	1
IV	Hsu	1.25 % acetic acid-CH_3OH (80:20)	Ambient	1.5	254	30°C	1
V	USP	0.05 M phosphate buffer pH 5.0-CH_3CN (96:4)	Ambient	1.5	230	30°C	1

*Wavelength used: 254 min.

substances **6** and **13** were obtained from Antibioticos and Biochemie S. A., Spain. The other related substances are decomposition products. Amoxicilloic acid (5S, 6R) (**4**) and amoxicilloic acid (5R, 6R) (**5**) were prepared as described by Munro.[16] The preparation of amoxilloic acid (5S) (**7**) and amoxilloic acid (5R) (**8**) will be described elsewhere. 2-Hydroxy-3-(4-hydroxyphenyl)pyrazine (**12**) was prepared in a similar way as described by Lebelle.[17] Amoxicillin (2R) piperazine-2,5-dione (**10**) and amoxicillin (2S) piperazine-2,5-dione (**11**) were prepared as described by Roets[18] and Haginaka.[19] 4-Hydroxyphenylglycylamoxicillin (**9**) was prepared in a similar way as described by Grant.[20] The oligomeroates (**14**) and oligomers (**15**) were prepared as described by Roets[18] and Bundgaard.[21]

Solvents and reagent

Acetonitrile (HPLC grade) was from Rathburn (Walkerburn, Scotland). Methanol (Roland, Brussels, Belgium) was distilled before use. Potassium dihydrogen phosphate, acetic acid were from Acros Chimica (Beerse, Belgium). Water was distilled twice.

Table 2

General Information on Columns

Columns	Stationary Phases	Particle Size (μm)
A, B*	Hypersil ODS (Shandon, Runcorn, UK)	5
C, D	Bio-Sil C_{18} (Bio-Rad, Nazareth, Belgium)	5
E, F*	Spherisorb ODS-1 (Phase Sep'n, Queensferry, UK)	10
G	RoSil C_8 (Alltech, Deerfield, IL, USA)	8
H	ChromSpher C_8 (Chrompack, Middleburg, Netherlands)	5
I, J	Zorbax C_8 (DuPont Co., Wilmington, DE, USA)	7
K	PRP-1 (Hamilton, Reno, NV, USA)	7-9
L, M*	PLRP-S (Polymer Laboratories, Church Stretton, UK)	8

* Columns used in laboratory B

LC Apparatus and Column

The equipment consisted of a L-6200 (Merck-Hitachi, Darmstadt, Germany), a Model CV-6-UHPa-N60 Valco injector (Houston, TX, USA), with a 20 μL loop or a 50 μL loop, a Model D 254 nm fixed-wavelength UV monitor (LDC/Milton Roy, Riviera Beach, FL, USA) and an integrator Model 3396 Series II (Hewlett-Packard, Avondale, PA). The columns (25 cm x 0.46 cm i.d.) used in this study are reported in Table 2. Most of the experiments are carried out in laboratory A using this equipment, but a number of experiments were repeated in laboratory B, to examine for reproducibility. The columns used in the laboratory B are identified with an asterisk. The equipment used in laboratory B was of similar quality.

Mobile Phase and Sample Preparation

The mobile phases were prepared as described in Table 1. For some columns, the amount of organic modifier was slightly adapted in order to obtain a similar retention time for amoxicillin.

In this study mobile phase was used as the solvent. For the selectivity study, the following concentrations were used: amoxicillin 1.2 mg/mL, **2** and **3**: 0.1 mg/mL, **4**, **5** and **6**: 0.2 mg/mL, **12**: 0.02 mg/mL, other related substances: 0.5 mg/mL, 20 μl of these solutions was injected.

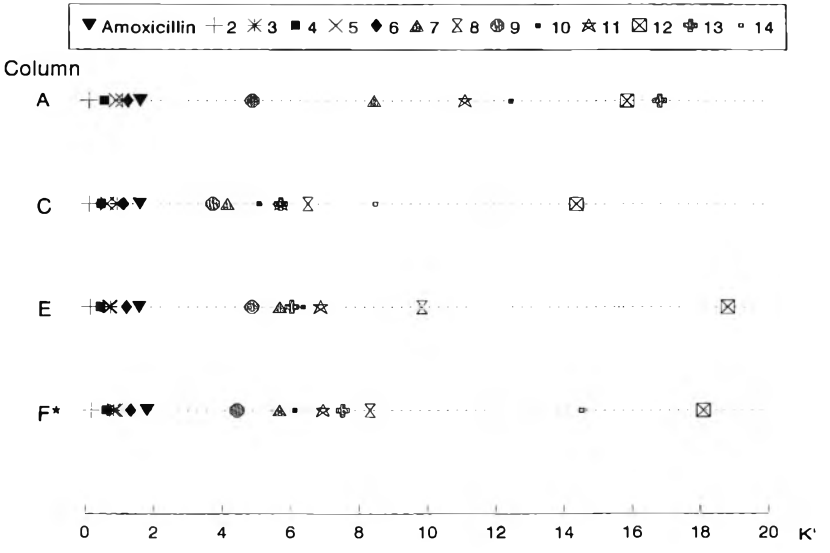


Figure 2. Capacity factors of amoxicillin and its related substances on C_{18} columns according to method I.

RESULTS AND DISCUSSION

Selectivity Study

During preliminary work in laboratory A, three different C_{18} stationary phases (A, C, E) were used for the selectivity study of methods I, II, IV and V and three different C_8 stationary phases (G, H, I) were used for the selectivity study of method III. Some experiments were carried out using poly(styrene-divinylbenzene) stationary phases. The trimeroates (**14**, n = 1) and the oligomers (**15**) of amoxicillin were always eluted far after amoxicillin, therefore they are not shown in the results of the isocratic experiments. Results for other related substances with capacity factors (k^*) of more than 20 are not shown either. The experiments were repeated on one column in laboratory B.

For method I, the results are shown in Fig.2. The concentration of methanol in the mobile phase was adjusted for each column (A = 6, C = 5.5, E = 2, F* = 5) in order to obtain similar retention times for amoxicillin. Amoxicillin was completely separated from related substances on all four

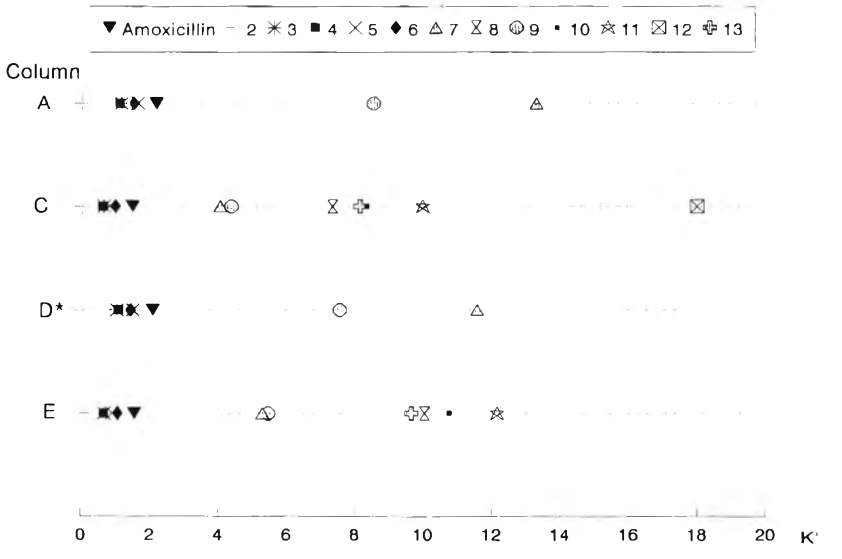


Figure 3. Capacity factors of amoxicillin and its relatied substances on C_{18} columns according to method 2.

columns. For method II, the results are shown in Fig.3. The concentration of methanol in the mobile phase was adjusted for each column (A = 5, C = 3.7, D* = 5.5, E = 3). Amoxicillin was completely separated from its related substances on the four columns.

Methods I and II are very similar, there are only small differences in the pH and the concentration of the buffer. For method III, the results are shown in Fig.4. The concentration of methanol in the mobile phase was adjusted for each column (G = 4, H = 2, I = 1.5, J* = 7.5). Amoxicillin was separated from its related substances on all four columns.

This method corresponds to method I, except that C_8 columns are used in method III. For method IV, the results are shown in Fig.5. The concentration of methanol in the mobile phase was adjusted for each column (A = 30, B* = 26, C = 10, E = 20). Amoxicillin was separated from its related substances on column E but not on columns A, B* and C.

In the figures above, it can be seen that for the same method, the sequence of related substances can be different on different columns, which is common for silica boned reverse phases.

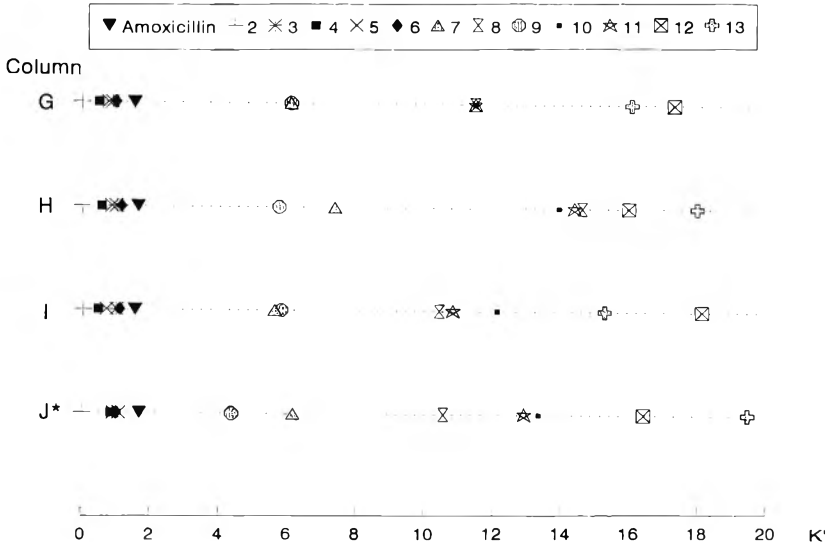


Figure 4. Capacity factors of amoxicillin and its related substances on C_8 columns according to method 3.

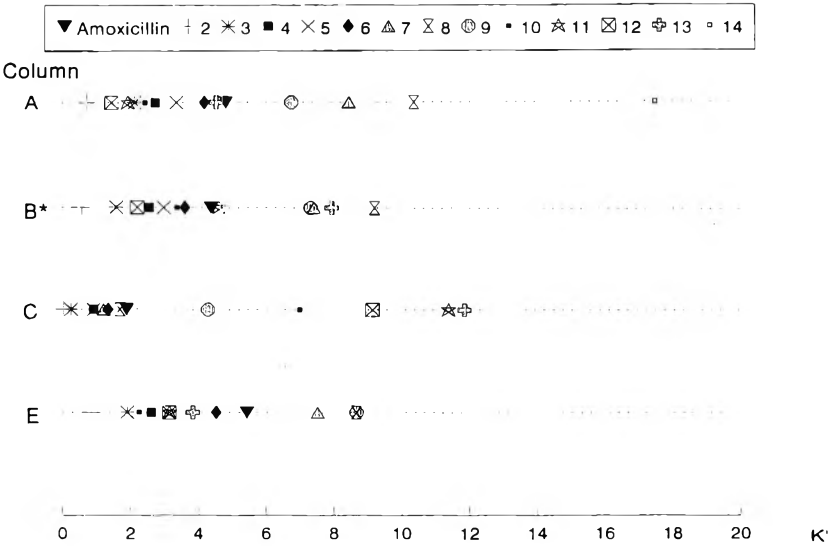


Figure 5. Capacity factors of amoxicillin and its related substances on C_{18} columns according to method 4.

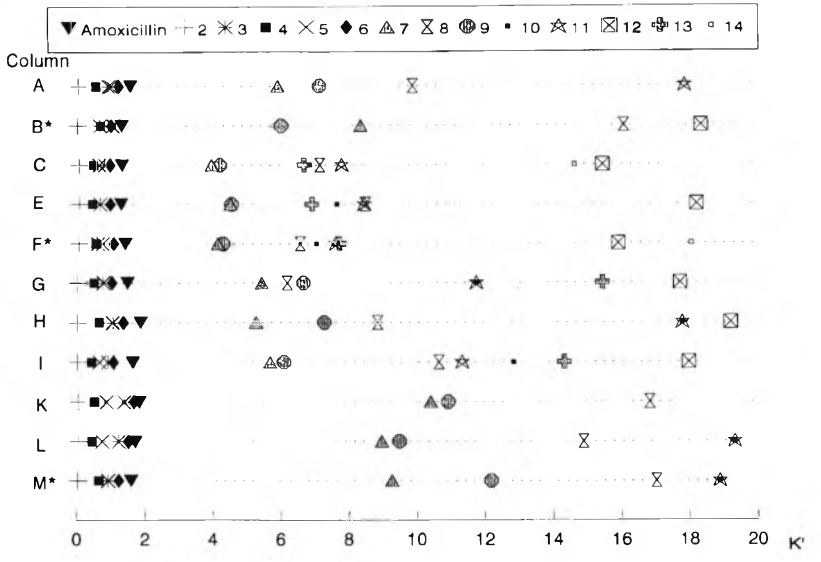


Figure 6. Capacity factors of amoxicillin and its related substances on different columns according to the USP method.

For the USP method, after preliminary work on three C_{18} columns in laboratory A it was clear that this method gave a satisfactory and repeatable selectivity. This was confirmed by laboratory B, using two C_{18} columns. The USP method is very similar to methods I and III, except that CH_3CN is used as the organic modifier instead of CH_3OH. As method V is a current official method, only this method was further examined with C_8 columns (G, H, I, J*) and even with poly(styrene-divinylbenzene) columns (K, L, M*) although methods I, II, III also gave satisfactory results. To improve the efficiency, columns K, L and M* were used at 50 °C instead of 30 °C as prescribed by the USP method. The results of the selectivity study are shown in Fig. 6, on all columns amoxicillin was separated from its related substances. The related substances were also reasonably well separated from each other but here also the sequence may be different on different columns.

The results on C_8 or C_{18} columns were similar. The separation from L-amoxicillin was somewhat less good on the polymer columns. Table 3 shows general information on method performance using the USP method. According to the USP monograph, the capacity factor of amoxicillin, k^*, must be between 1.1 and 2.8, the column efficiency not less than 1700 theoretical plates, the symmetry factor not more than 2.5. So all the parameters comply with the requirements of the USP method.

Table 3

General Information on Method Performance Following USP Method

Column	Content (%) of Acetonitrile in Mobile Phase	k′ Amoxicillin	S Amoxicillin	n Amoxicillin	Rs Amoxicillin Cefadroxil	α	α′
A	2.8	1.61	1.18	9690	9.2	1.68	1.28
B*	3.0	1.35	0.90	10240	7.7	1.73	1.31
C	2.0	1.34	1.25	3020	2.2	1.30	1.37
E	2.0	1.30	1.08	2160	2.5	1.42	1.33
F*	4.0	1.43	1.10	5670	3.5	1.29	1.31
G	2.0	1.50	1.10	4880	4.0	1.38	1.43
H	0.6	1.89	1.22	5830	5.7	1.56	1.36
I	1.0	1.66	1.19	4730	3.3	1.34	1.53
K	0.8	1.85	2.04	1710	4.9	1.91	1.09
L	0.8	1.73	1.50	3840	7.1	1.92	1.14
M*	0.8	1.60	1.30	2667	8.2	2.64	1.29

* Columns used in laboratory B

k′ = capacity factor; S = symmetry factor; n = number of theoretical plates; Rs = resolution.

$\alpha = k'_{\text{cefadroxil}} / k'_{\text{D-amoxicillin}}$; $\alpha' = k'_{\text{D-amoxicillin}} / k'_{\text{L-amoxicillin}}$

Resolution Test

A resolution test using cefadroxil was developed. The structure of cefadroxil is close to that of amoxicillin, therefore its chromatographic behaviour is related to that of amoxicillin. Cefadroxil has been used as an internal standard for the assay of amoxicillin.[2] The results for the resolution test are shown in Table 3. The resolution between cefadroxil and amoxicillin is more than 2.0 for all columns. It was preferred to use cefadroxil instead of L-amoxicillin, because the latter is not commercially available.

Related Substances Test

A related substances test which is based on the USP method was also developed. Considering the nature of the potential impurities, it is necessary to use gradient elution. The chromatographic procedure was carried out with mobile phase A: 0.05 M phosphate buffer pH 5.0-CH_3CN (99:1) and B: 0.05 M phosphate buffer pH 5.0-CH_3CN (80:20). A freshly prepared test solution with a concentration of 1.5 mg/mL was injected with a 50 µL loop. The elution was started isocratically with ratio A:B of 92:8. After 8 min., a linear gradient

Table 4

Capacity Factors of Amoxicillin and Its Related Substances on Three C_{18} Columns as Obtained by Gradient Elution

	k′ Columns		
	A	**C**	**E**
1	1.9	1.9	1.3
2	0.2	0.1	0.2
3	1.3	1.1	0.7
4	0.6	0.5	0.5
5	1.1	0.8	0.7
6	1.5	1.4	1.0
7	6.9	4.6	4.2
8	7.9	6.2	6.4
9	6.3	5.1	4.2
10	7.9	6.2	6.1
11	7.6	6.5	6.1
12	9.8	8.8	8.0
13	9.1	6.2	6.1
14 (n=0)	7.9	7.6	7.6
14 (n=1)	10.8	9.8	9.7
15 (n=0)	10.4	9.1	9.7
15 (n=1)	11.7	10.5	10.9

Mobile phase: A: 0.05 M phosphate buffer, pH 5.0-CH_3CN (99:1)
B: 0.05 M phosphate buffer, pH 5.0-CH_3CN (80:20)

Gradient elution: 0 to 8 min., isocratic elution with ratio A:B of 92:8; 8 to 22 min., a linear gradient elution to ratio A:B of 0:100; 30 to 45 min., isocratic elution with ratio A:B of 0:100; 45 to 60 min., isocratic elution with A:B of 92:8.

elution was started to reach a mobile phase ratio A:B of 0:100 over a period of 22 min. The chromatography was continued with mobile phase B during 15 min. Then the column was equilibrated with the originally chosen mobile phase during 15 min. The related substances test was examined using three

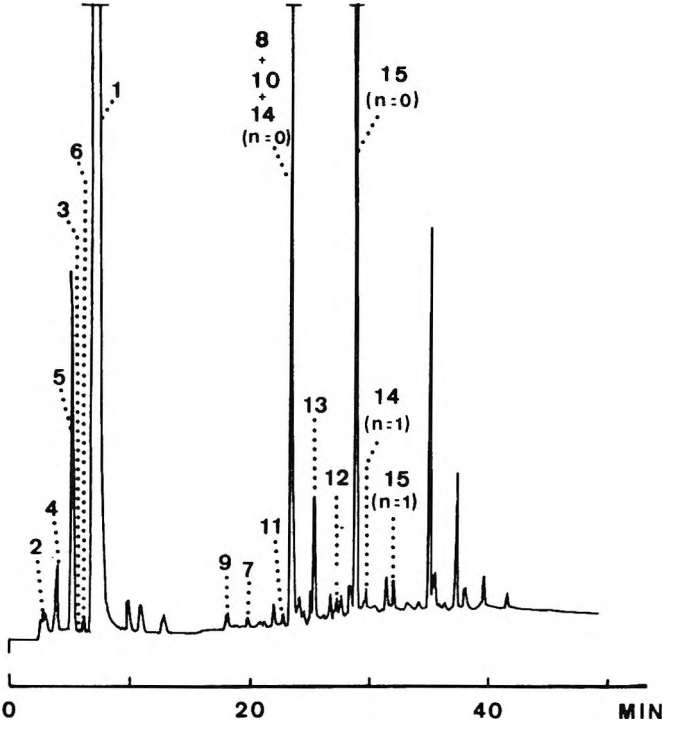


Figure 7. Typical chromatogram of amoxicillin sodium on Hypersil C_{18} (column A) with gradient elution. Mobile phase: A: 0.05 M phosphate buffer pH 5.0-CH_3CN (99:1), B: 0.05 M phosphate buffer pH 5.0-CH_3CN (80:20). Gradient elution: 0 to 8 min, isocratic elution with ratio A:B of 92:8; 8 to 22 min, a linear gradient elution to ratio A:B of 0:100; 30 to 45min, isocratic elution with ratio A:B of 0:100; 45 to 60 min, isocratic elution with A:B of 92:8.

C_{18} columns (A, C, E). The results are shown in Table 4. The results show the good selectivity of the gradient method for related substances. Column E gave a less good separation of related substances than columns A and C. Therefore it seems better to use only C_{18} columns with particle size of 5 μm. A typical chromatogram of an old sample of amoxicillin sodium obtained with column A is shown in Fig. 7.

Linearity, Repeatability and Stability

The quantitative aspects of this method have been examined. For linearity

amounts corresponding to 20, 30 or 40 µg of amoxicillin were analysed. The total number of analyses was 18. This yielded a calibration curve: $y = 557200x + 171000$, with y = peak area, x = amount injected in µg and with the correlation coefficient $r = 0.9999$ and the standard error of estimates $S_{y,x} = 46900$.

The repeatability was checked by analysing the same solution corresponding to 30 µg of amoxicillin six times. The relative standard deviation (RSD) for the peak area of amoxicillin was 0.15 %.

The stability at 22 °C of a solution (1.5 mg/mL) of amoxicillin trihydrate or amoxicillin sodium in the mobile phase A was examined. The solutions were injected every two hours over a period of 16 hours. No decrease of the peak area of amoxicillin was observed and the RSD values for the mean were 0.33 % (n = 8) and 0.25 % (n = 9) for the trihydrate and sodium salt, respectively. It was concluded that amoxicillin remained stable in the mobile phase A for at least 16 h.

Limit of Detection and Limit of Quantitation

For the related substances test, it was decided to inject 50 µL of a solution containing 1.5 mg of amoxicillin per mL. For this quantity, the limit of detection (LOD) was 0.02 % with a signal to noise ratio of 7. The limit of quantitation (LOQ) was 0.05 % (n = 6, RSD = 10 %).

CONCLUSION

It can be concluded that the USP method is one of the isocratic methods that is sufficiently and reproducibly selective to be used for the assay of amoxicillin. Cefadroxil may be used in the resolution test. The gradient elution method, based on the USP method, seems suitable as a related substances test.

The performance of the USP method and the related substances test derived from it will be further examined in a collaborative study.

ACKNOWLEDGEMENT

The National Fund for Scientific Research (Belgium) is acknowledged for financial support.

The authors thank Antibioticos and Biochemie S.A., Barcelona, Spain, for the gift of some of the related substances and I. Quintens for editorial assistance.

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Received December 7, 1995
Accepted December 20, 1995
Manuscript 4047

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1909–1915 (1996)

SIMULTANEOUS DETERMINATION OF HONOKIOL AND MAGNOLOL IN MAGNOLIA OFFICINALIS BY CAPILLARY ZONE ELECTROPHORESIS

Cynthia Y. C. Chou[1,2], T. H. Tsai[3],
M. F. Lin[1], C. F. Chen[2,3]*

[1]Department of Pharmacy
Veterans General Hospital-Taipei
Taipei 11217, Taiwan
[2]Institute of Pharmacology,
National Yang-Ming University
Taipei 11221, Taiwan
[3]Department of Pharmacology,
National Research Institute of Chinese Medicine,
Taipei 11221, Taiwan.

ABSTRACT

A simple and rapid capillary electrophoretic method was developed for the simultaneous determination of honokiol and magnolol in *Magnolia officinalis* extracts and dextrorphan was used as the internal standard. The running buffer was composed of 22.5 mM Na_2HPO_4 and 10 mM $Na_2B_4O_7$ (pH 9.1-9.2). The linear calibration range was 2-20 μg/mL for honokiol and 5-50 μg/mL for magnolol. It was found that 0.95 ± 0.02 mg of honokiol and 4.37 ± 0.08 mg of magnolol were contained in the ethanol (50%) extracts of 1 g of *Magnolia officinalis*. The contents of these two active principles in *Magnolia officinalis* was successfully determined within 12 min.

INTRODUCTION

The stem bark of *Magnolia officinalis* (Hou-Po) has been used as a folk medicine in China for the treatment of thrombotic stroke, typhoid fever and headache.[1] It has been reported that Hou-Po possesses antimicrobial activities[2] and central depressant effects.[3] Honokiol and magnolol (Fig. 1), isomers of neolignans, have been isolated from the bark of this plant and other Magnoliaceae.[4] These compounds inhibit intracellular calcium mobilization in platelets,[5] relax vascular smooth muscle,[6] inhibit collagen-induced platelet serotonin release[7,8] and have antihemostatic and antithrombotic effects.[9]

Recent studies indicated that magnolol has antiinflammatory and analgesic effects,[10] and modulates central serotonergic activities.[11] These two compounds are also effective in inhibition of 11 beta hydroxysteroid dehydrogenase,[12] acetyltransferase[13] and hydroxyl radicals,[14,15,16] and have antiemetic activities.[17] Several methods for the determination of honokiol and magnolol have been described, including ion pair HPLC,[18] HPLC photodiode array detection,[19] and liquid chromatography-mass spectrometry.[20,21] However, none of the methods has been described to the determination of honokiol and magnolol by capillary electrophoresis.

In this work, we developed a simple and rapid capillary zone electrophoretic method, using dextrorphan as the internal standard, for the simultaneous determination of these compounds in *Magnolia officinalis*. The proposed technique is a viable alternative to HPLC and should be useful for the quality control of *Magnolia officinalis*.

MATERIALS AND METHODS

Materials and reagents

Magnolia officinalis was purchased from a traditional Chinese herbal drug store in Taipei. Authentic honokiol and magnolol were obtained from Nacalai Tesque (Kyoto, Japan), disodium hydrogen phosphate, sodium tetraborate, ethanol (99.5%) and NaOH from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore Corporation, Bedford, MA, U.S.A.) was used for all preparations.

Extraction

A 5 g powder of *Magnolia officinalis* was boiled with 50 mL of extraction solvents [water, ethanol (50%), 0.1 M NaOH] for 5 min. Extraction was repeated twice. The two extracts were combined and diluted to 100 mL in a

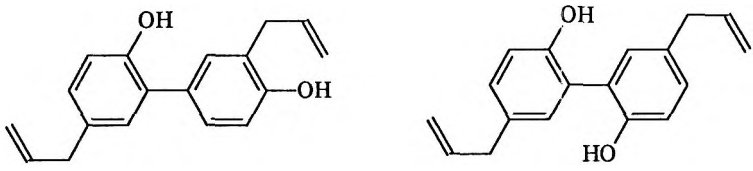

Figure 1. Chemical structures of honokiol (left) and magnolol (right).

volumetric flask. A 1 mL aliquot of this solution was filtrated using 0.2 μm disposable syringe filters, followed by adding a known concentration of dextrorphan (20 μg/mL) as internal standard. A 30 nL (5 sec pressurized injection) of this reconstituted sample was injected into the capillary electrophoresis system directly.

Apparatus andCondition

All measurements were performed on a Beckman P/ACE 2200 (Fullerton, CA, U.S.A.) capillary electrophoresis system, equipped with a UV detector set at 214 nm. A 75 μm ID uncoated fused-silica capillary of 57 cm total length (Polymicro Technologies, Phoenix, AZ, U.S.A.), was employed. Sample injection was done by introducing a pressure of 0.5 Psi for 5 sec to the sample. The applied voltage was a constant 15 kV (positive to negative polarity), the temperature was set at 25 °C and the running time was 12 min. The electrophoresis buffer was 22.5 mM disodium hydrogen phosphate and 10 mM sodium tetraborate buffer (pH 9.1-9.2). Prior to each run, the capillary was rinsed for 2 min with running buffer. After each run, the capillary was washed for 3 min with 0.1 M NaOH followed by deionized water.

Determination of Honokiol and Magnolol

Calibration graphs for 4 concentrations of honokiol (2, 5, 10, 20 μg/mL) and 4 concentrations of magnolol (5, 10, 20, 50 μg/mL) were analysed by capillary electrophoresis. The contents of honokiol and magnolol in the extract of *Magnolia officinalis* were determined from the peak area ratio by using the equation for linear regression from the calibration curve.

RESULTS AND DISCUSSION

The structures of honokiol and magnolol suggested that could be analysed as anions. We found that a buffer solution containing 22.5 mM Na_2HPO_4 and

Table 1

Intra- and Inter-Assay Precision and Accuracy in Honokiol and Magnolol Determination (n=5)

	Nominal Concentration (μg/mL)					
	Honokiol			Magnolol		
	2	10	20	5	20	50
Intra-Assay						
Mean	2.15	9.86	20.07	4.92	20.23	49.92
S. D.	0.12	0.14	0.06	0.25	0.47	0.15
% C. V.	5.7	1.4	0.3	5.0	2.3	0.3
Accuracy (%)	7.6	-1.3	0.3	-1.6	1.1	-0.2
Inter-Assay						
Mean	1.76	10.11	19.20	5.505	19.92	50.01
S. D.	0.19	0.24	0.12	0.31	0.55	0.15
% C. V.	10.7	2.4	01.6	6.2	2.8	0.3
Accuracy (%)	-11.8	1.1	-0.5	1.0	-0.3	0.0

Precision (% C. V.) = [standard deviation (S. D.) / mean concentration] x 100.
Accuracy (%) = (mean conc. - actual conc.) / actual conc.] x 100.

10 mM $Na_2B_4O_7$ (pH 9.1-9.2) could separate these two compounds without interference with other peaks. Fig. 2 (A) shows typical electropherogram of the standard mixtures. Fig. 2 (B) shows the ethanol (50%) extracts of *Magnolia officinalis*. The migration times of internal standard (dextrorphan), honokiol and magnolol were found to be 4.8, 6.4, 8.5 min, respectively. All measurements were completed within 12 min.

The equations of the calibration curve for honokiol and magnolol were $y = 5.5929x - 0.0411$ ($r^2 = 0.999$) and $y = 6.6869x + 0.2178$ ($r^2 = 0.999$), respectively. Where x is the response in peak area ratio and y is the amount of compound analyzed. The linearity ranges were 2-20 μg/mL for honokiol and 5-50 μg/mL for magnolol. The lower detection limits for honokiol and magnolol, at a signal-to-noise ratio of 3, were 0.2 and 0.5 ng, respectively.

The reproducibility of the method can be defined by examining both intra-assay and inter-assay variabilities. Table 1 shows the intra- and inter-assay precision and accuracy in honokiol and magnolol determination (n=5). Table 2

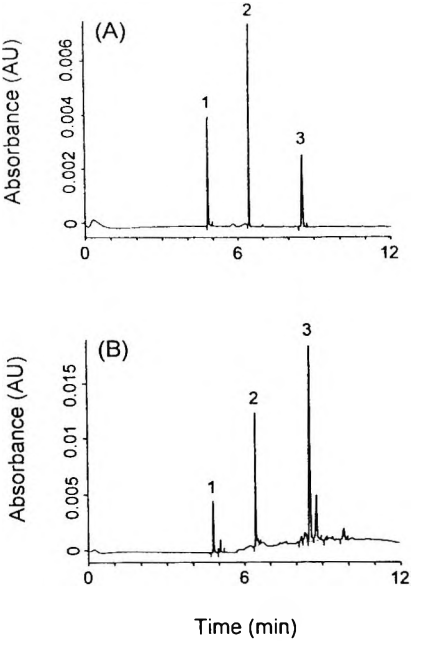

Figure 2. (A) Capillary electropherogram of a standard mixture. (B) Capillary electropherogram of the ethanol (50%) extracts of *Magnolia officinalis*. 1: dextorphan (internal standard); 2: honkiol; 3: magnolol.

Table 2

Contents of Honokiol and Magnolol in Different Extracts of 1 g of Magnolia Officinalis (mg/g)

Extraction Solvent	Honokiol	Magnolol
Water	0.25 ± 0.01	0.98 ± 0.02
Ethanol (50%)	0.95 ± 0.02	4.37 ± 0.08
0.1M NaOH	3.68 ± 0.09	27.49 ± 0.43

Results are mean ± S. D. (n=6)

gives the contents of honokiol and magnolol in extracts of *Magnolia officinalis* obtained with different solvents. It appears that 0.1 M NaOH is the best solvent for the extraction of honokiol and magnolol. In conclusion, the proposed

technique is suitable for the simultaneous determination of honokiol and magnolol by capillary zone electrophoresis, and should be useful for the quality control of *Magnolia officinalis*. The short analysis time, the small amount of samples and easily cleaned column, make this method a potential alternative to HPLC.

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Received January 7, 1996
Accepted January 31, 1996
Manuscript 4060

J. LIQ. CHROM & REL. TECHNOL., 19(12), 1917-1926 (1996)

A STUDY ON THE POSSIBILITY OF USING HPLC FOR THE DETERMINATION OF 2,4-D IN TOMATOES

V. Gökmen, J. Acar
Department of Food Engineering
Hacettepe University
TR 06532 Beytepe, Ankara, Turkey

ABSTRACT

An HPLC method for the determination of 2,4-D in tomatoes has been studied. Tomato samples were extracted with acetone-dichloromethane (2:1) after hydrolysis. The extract was cleaned up with acid-base partition and furthermore eluting through XAD-2 column. Then, it was analyzed using reverse phase HPLC. Four different mobile phase mixtures, at two different flow rates of 1.0 and 1.5 mL/min, were tried, to choose the best conditions in the final HPLC determination step. The best results were obtained using the mixture of acetonitrile:water, containing 2% of acetic acid (50:50, v/v), at a flow rate of 1.0 mL/min for the separation of 2,4-D standard solutions, but the baseline separation of 2,4-D in the final solution of sample extract could not be achieved due to interferences causing poor resolution as well as low recovery.

INTRODUCTION

Although 2,4-dichlorophenoxyacetic acid (2,4-D) is a herbicide widely used for almost 50 years in modern agriculture,[1-3] it is also known as a plant growth regulator.[4-7] The effect of 2,4-D on fruit setting and development of greenhouse-grown tomatoes is well known for a number of years.[8,9]

2,4-D is allowed to be used only as a herbicide in Turkey, but it is suspected that some local producers use it as a growth regulator to stimulate tomato fruit set in greenhouses during mild winter conditions. The maximum permitted concentration of 2,4-D established by Food and Agricultural Organization/World Health Organization (FAO/WHO) in various foods is in the range of 0.05-0.5 ppm.[10] Because of uncertainty of the carcinogenic effect of 2,4-D, there is a strong pressure towards the abolishment of its use.

For the residual analysis of 2,4-D in plant materials, gas chromatography (GC), with electron capture detection, is mainly used.[11-15] However, because of the high polarity of 2,4-D in its acid form, it has to be derivatized first for GC analysis.[2,16-19] Various derivatization procedures have been developed for the determination of acidic residues to render them volatile. In doing so, additional analysis time, expense and, sometimes, errors due to non-reproducible results are introduced by derivatization, making the method unattractive to many chemists.[20] Methylation has been the method of choice for a number of years, because the reaction is rather simple with few side products.[16] High-performance liquid chromatography (HPLC) can also be used for the separation of 2,4-D in its underivatized free acid form.[16,19,21-29] However, no residue method for the determination of 2,4-D or any other compounds having similar chemical structure in plant materials has appeared in the literature using HPLC.

This study was carried out to investigate the possibility of using HPLC in place of GC for the determination of 2,4-D residues in tomatoes.

EXPERIMENTAL

Material

Tomato samples used throughout the recovery trials were all 2,4-D free and kept in a deep freezer until just prior to analysis.

Reagents

2,4-D, hexadecyltrimethylammonium bromide (cetrimide), K_2HPO_4 and NaH_2PO_4, $NaHCO_3$, NaCl, NaOH, H_2SO_4 and anhydrous Na_2SO_4 were all reagent grade and obtained from Merck. All solvents used were of HPLC grade (Merck) and water was bidistilled.

Cetrimide was dissolved in methanol to a concentration of 0.03 M and $NaHCO_3$ was dissolved in 80% acetonitrile in water to a concentration of 0.1 M. All other solutions used throughout the experiments were prepared in bidistilled water.

Anhydrous Na_2SO_4 was dried at 550°C for 2 hrs. Filter papers were extracted with dichloromethane before use.

Mobile Phases

In order to optimize the chromatographic conditions for the separation of 2,4-D, its capacity factor on a C_{18} column was determined with three different mobile phase compositions, at two flow rates of 1.0 and 1.5 mL/min. Mobile phase compositions used were as follows:

I. Methanol:water (75:25, v/v), containing 0.001 M PO_4^{-3} and 0.005 M cetrimide.

II. Acetonitrile:water, containing 2% of glacial acetic acid (75:25, v/v).

III. Acetonitrile:water containing 2% of glacial acetic acid (50:50, v/v).

Mobile phase mixtures were passed through a 0.45 μm regenerated cellulose acetate membrane (Sartorius) and degassed ultrasonically.

2,4-D Standard Solutions

Stock solution of 2,4-D, at a concentration of 1000 μg/mL, was prepared, both in methanol and in the mixture of isopropanol:water (75:25, v/v). Working standard solutions of 0.5, 1.0, 2.0, 3.0 and 4.0 μg/mL were prepared, appropriately diluting the stock solutions with methanol for mobile phase I and with the mixture of isopropanol:water (75:25, v/v) for mobile phases II and III to improve the separation. These solutions were kept at 4°C just prior to analysis.

High-Performance Liquid Chromatography

An HPLC system, consisting of Varian 9010 solvent delivery system, Varian 9050 variable wavelength UV-VIS detector, Rheodyne 7161 six way injector, equipped with a 10 μL sample loop was used. The MicroPak® column, made of stainless steel (150 x 4.0 mm I.D.), packed with octadecyl groups (C_{18}, 5μm), was operated at ambient temperature. It was protected with a microparticulate guard column (40 x 4.0 mm I.D.).

UV Spectra

Working standard solutions in the mixture of isopropanol:water (75:25, v/v) were used to record UV spectra for 2,4-D on a Shimadzu 2101 UV-Vis spectrophotometer.

Sample Preparation

Tomatoes were blended in a Waring blender. 15 mL of 1 N NaOH and 60 mL of water were added into 25 g of blended tomato sample and the mixture was hydrolyzed in a water bath at 95°C for 2 hrs. After cooling, the amount of water evaporated during hydrolysis was added and the mixture was acidified, adding 5 mL of 20% H_2SO_4. Then it was homogenized with 200 mL of acetone for 2 min, at medium speed, in a Virtis homogenizer and filtered through a black band filter paper into a graduated cylinder. The filtrate volume was recorded (V_f) and the filtrate was transferred into a 1000 mL separatory funnel.

It was then saturated with V_f/10 g of NaCl, shaking vigorously for 3 min. 100 mL of dichloromethane was added and the filtrate was extracted by shaking for 2 min. After phase separation, the aqueous phase was discarded. The organic phase was quantitatively transferred into a flask containing 25 g of anhydrous Na_2SO_4 and dried for 20 min. The organic phase was then filtered through a black band filter paper into a 1000 mL separatory funnel and extracted with 100 and 50 mL of 0.5 N NaOH. Combined NaOH extracts were acidified with 20% H_2SO_4 (pH≤1.0).

The extract was cleaned up by eluting through Amberlite® XAD-2 column at a flow rate of 5 mL/min, and eluate was discarded. The column was then washed with water to neutralize the eluate. The column was eluted with 0.1 M $NaHCO_3$ in 80% acetonitrile in water at a flow rate of 2.0 mL/min and the eluate was collected in a flask. The eluate was acidified with 20% H_2SO_4 (pH≤ 1.0) and transferred into 250 mL separatory funnel. It was extracted with 2x50 mL of dichloromethane.

Combined dichloromethane extracts were filtered through a black band filter paper, covered with a layer of anhydrous Na_2SO_4 into a 250 mL round bottom flask. It was evaporated to dryness in a rotary evaporator at 40°C. Furthermore, residue was dried under a gentle stream of nitrogen. Dried residue was redissolved immediately in the mixture of isopropanol:water (75:25, v/v). 10 μL of this solution was injected into the HPLC column.

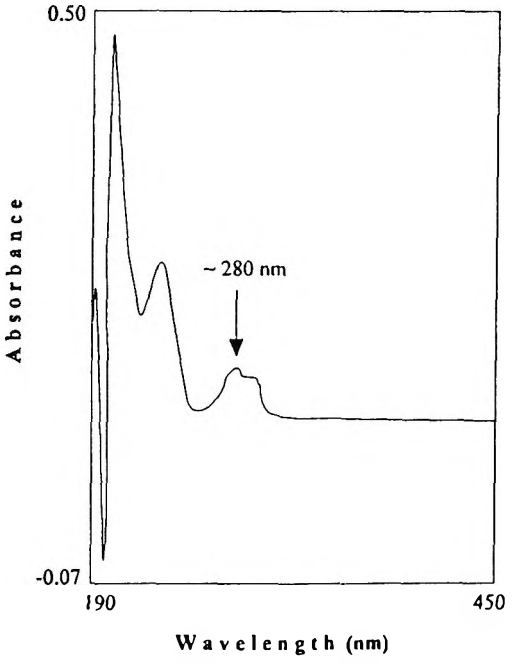

Figure 1. UV spectrum of 2,4-D.

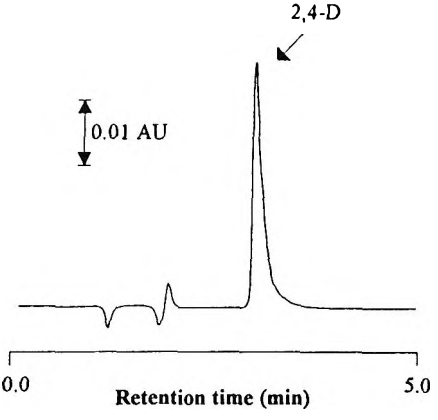

Figure 2. Separation of 2,4-D on C18 column. Chromatographic conditions: mobile phase: mixture of acetonitrile:water containing 2% of glacial acetic acid (50:50, v/v), flow rate: 1.0 mL/min; λ=280 nm.

Calculation of the Results

Corresponding sample amount in the extract was calculated by the following formula:

$$m = \frac{S \times V_f}{T}$$

where,

m = amount of sample in the extract, g
S = initial weight of sample, g
V_f = volume of filtrate, mL
T = total theoretical volume of filtrate, mL (200 mL acetone + 60 mL water + 15 mL 1 N NaOH + 5 mL 20% H_2SO_4 + water in tomato sample)

The volume contraction of acetone and water must be taken into account when determining total theoretical volume of the filtrate.

Concentration of 2,4-D in the final solution was calculated using the calibration curve prepared daily, based on concentration vs. peak height. The amount of 2,4-D in tomato was then calculated as follows:

$$2,4-D \text{ in tomato } (\mu g / g) = \frac{C_f \times V}{m}$$

where,

C_f = concentration of the final solution, μg/mL
V = volume of the final solution, mL
m = amount of sample in the extract, g

RESULTS AND DISCUSSION

Specific wavelength of 2,4-D for maximum absorbance was determined to be 280 nm, in accordance with the result of Roseboom et al. (1982). Figure 1 illustrates the typical spectrum of 2,4-D. Detection wavelength during HPLC analysis of 2,4-D was therefore set at 280 nm to obtain maximum sensitivity.

Mobile phase III, at a flow rate of 1.0 mL/min was found to be more efficient for the separation of 2,4-D on the C_{18} column. Therefore, it was chosen as the mobile phase for the residual analysis of 2,4-D in tomato. Symmetrical peaks and reproducible results were obtained for 2,4-D on the C_{18} column when mobile phase III at flow rate of 1.0 mL/min was used (Fig. 2). Correlation coefficient ® for the calibration graph of 2,4-D, based on

Table 1

Capacity Factors (k') Obtained on a C_{18} Column

Mobile phase No.	Capacity factor (k') 1.0 mL/min	1.5 mL/min
I	2.88	2.81
II	0.63	0.65
III	2.38	4.32

concentration vs. peak height for mobile phase III at flow rate of 1.0 mL/min, was 0.998 (n=5). Band broadening was observed for mobile phase I, while separation of 2,4-D was not good for mobile phase II. The capacity factors (k'), obtained for three mobile phase compositions at two flow rates of 1.0 and 1.5 mL/min, are given in Table 1.

The extraction procedure applied in this study uses acetone/ dichloromethane extraction after alkaline hydrolysis. Alkaline hydrolysis is thought to be necessary to convert the bound and conjugated residues of 2,4-D present in tomato to free acid residue.[13,17] Percent recoveries obtained were 92.1%, 71.4% and 68.8% for the corresponding spiking levels of 0.5, 2 and 4 µg/g of 2,4-D, respectively. These recoveries are lower than the recoveries recorded in many GC methods.[3,13,14] Although 2,4-D could be sensitively detected as the standard solution by HPLC, it could not be possible to analyze the residues of 2,4-D in tomato samples sensitively due to interferences.

Many of the colored compounds co-extracted from tomato could be removed by acid-base partition. However, the final extract still contained colored interferences after clean-up by eluting through an XAD-2 column. These co-extractives from the tomato matrix adversely affected the separation of 2,4-D on the C_{18} column. Baseline separation could not be achieved, thus preventing proper integration of the resulting chromatograms. Figure 3 illustrates the chromatogram of tomato extract spiked with 0.5 µg/g of 2,4-D.

CONCLUSION

It was thought, in the beginning of this study, that an HPLC method for the residual analysis of 2,4-D in tomato might be useful with some advantages over GC methods, such as ease of operation, economy and rapidity. However, sensitivity achieved was not found to be sufficient from the residual analysis considerations. Co-extractives from tomato matrix were the main problem preventing the separation of 2,4-D on a C_{18} column.

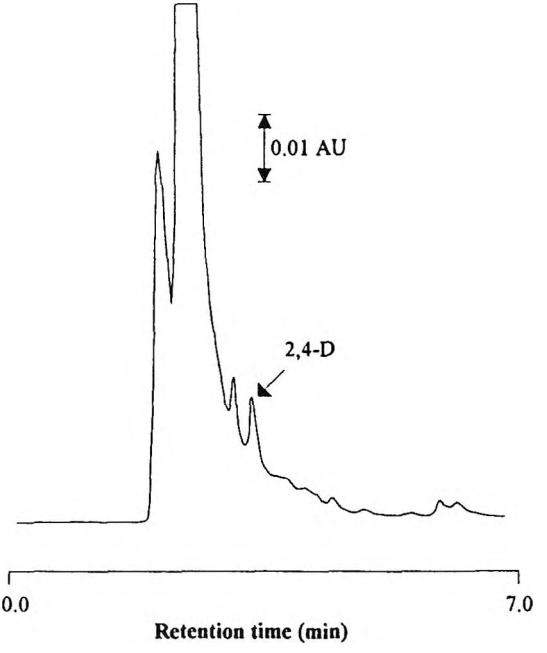

Figure 3. Chromatogram of tomato extract (spiking level=0.5 μg/g). (Chromatographic conditions are same as given in Fig. 2).

Clean-up, using acid-base partition and elution through the XAD-2 column, proven to be efficient in many GC methods appearing in the literature, was not found applicable in this HPLC method. It is thought that a clean-up procedure enabling removal of the interferences causing problems for the separation of 2,4-D in a C_{18} column must be improved.

The chromatographic conditions, proven to be efficient for the separation of 2,4-D as standard solutions in this study, may be used for monitoring the purity of 2,4-D formulations. Also, it may be possible to detect the residues of 2,4-D by HPLC in uncomplicated sample matrices such as water from agricultural areas where 2,4-D is widely used.

ACKNOWLEDGMENT

The authors would like to thank Hacettepe University Research Fund for financial support.

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Received September 12, 1995
Accepted November 8, 1995
Manuscript 3967

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1927–1935 (1996)

STEREOSELECTIVE DETERMINATION OF FLUOXETINE AND NORFLUOXETINE ENANTIOMERS IN PLASMA SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Simona Pichini, Roberta Pacifici, Ilaria Altieri,
Manuela Pellegrini, Piergiorgio Zuccaro

Clinical Biochemistry Department
Istituto Superiore di Sanita'
V. le Regina Elena 299
00161 Rome, Italy

ABSTRACT

The simultaneous liquid chromatographic determination of the enantiomers of both fluoxetine and its metabolite norfluoxetine in plasma samples of treated patients is described. The compounds are subjected to solid phase extraction before chromatography. The separation of the analytes is achieved using two chiralcel ODR columns on-line coupled and a mobile phase consisting of acetonitrile-$NaClO_4$ 0.3M (66/34 v/v, pH 2.5) at a flow rate of 0.6 mL/min. The compounds were detected by ultraviolet absorbance at 220 nm. The limit of quantification for each compound was 10 ng/mL.

INTRODUCTION

Fluoxetine (FLU) (± N-methyl-3-phenyl-3-[α,α,α-trifluoro-p-tolyl)oxy]-propylamine) (Fig. 1) and its N-demethylated metabolite norfluoxetine (N-FLU) are antidepressant drugs. Their activity is based on the selective inhibition of 5-

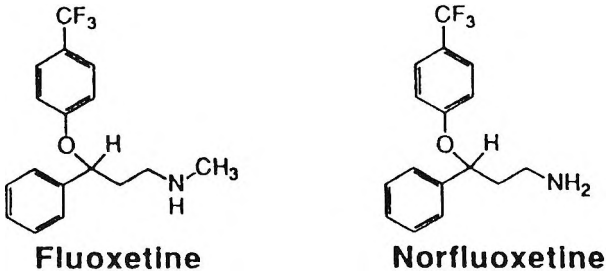

Figure 1. Structures of fluoxetine and norfluoxetine.

hydroxytryptamine (5-HT) recapture in the presynaptic neurons of the central nervous system.[1,2] Fluoxetine is sold, at present, as a racemate, despite different time courses in 5-HT uptake inhibition and rates of metabolism of fluoxetine R and S forms have been shown.[3-5] Similarly, (S)-N-FLU appears to be more potent than (R)-N-FLU in the inhibition of 5-HT uptake in vitro.[6]

Several chromatographic methods have been developed for the determination of fluoxetine and norfluoxetine in plasma or serum after administration of the parent drug,[7-13] but only two recent assays provide the measurement of the enantiomers of both fluoxetine and its metabolite, using chiral derivatization.[14-15]

In this paper we describe an enantioselective high performance liquid chromatographic (HPLC) method to determine the stereoisomers of fluoxetine simultaneously with the stereoisomers of norfluoxetine using solid phase extraction and spectrophotometric detection. The method was applied to plasma samples of treated patients using clozapine as an internal standard.

MATERIAL AND METHODS

Chemicals

Pure standards of (R, S)-FLU · HCl, (R, S)-N-FLU · HCl, (R)-N-FLU-HCl were provided from Eli Lilly Pharmaceuticals (Indianapolis, USA). The (R)-enantiomer of fluoxetine was obtained in our laboratory as described afterwards. Clozapine was provided from Sandoz S.p.A (Milano, Italy).

Extrelut-3 extraction columns were from Merck (Bracco, Milan, Italy). All solvents were of analytical reagent grade.

Chromatographic Instrumentation and Conditions

The HPLC system consisted of a Merck-Hitachi L6200 intelligent pump, a Merck-Hitachi L4200 UV-VIS detector set to 220 nm and a Merck-Hitachi D2000 chromato-integrator (Bracco, Milan, Italy). The columns used, from Daicel, Inc. (Schilling, Milan, Italy) were two Chiralcel ODR, both 25 cm x 4.6 mm i.d. and 10 μm particle size, on-line coupled with the second column heated at 40°C.

Resolution of the substances was achieved with acetonitrile-$NaClO_4$ 0.3M (66/34 v/v) containing 0.5 % triehylamine (pH 2.5 with perchloric acid). The mobile phase was left to equilibrate at least 2 hours before injections.

Isolation of the (R)-Enantiomer of Fluoxetine

In order to collect a purified enantiomer of fluoxetine, three Chiralcel ODR columns were on-line coupled, using the same mobile phase of the analytical separation.

This coupling permitted a 5 min interval between the peak baselines of the two fluoxetine stereoisomers, allowing the collection of the purified last-retained stereoisomer.

Subsequently, the stereoisomer dissolved in the mobile phase, alkalinized with NaOH 1N, was extracted with the same volume of n-hexane-isoamyl alcohol (97:3); the organic phase was evaporated under nitrogen and redissolved in 2 mL physiologic solution.

The absolute configuration of the extracted stereoisomer was determined comparing the optical rotation of its hydrochloride salt, measured with a Perkin Elmer 241 polarimeter (Perkin Elmer, Monza, Italy), to data reported in the literature.[4] It resulted to be the (R)- enantiomer of fluoxetine.

Sample Collection

Plasma samples were obtained from ten subjects treated for major depression with (R,S)-fluoxetine (Prozac). These participants received 20 mg of (R,S)-FLU once daily, usually taken between 7:00 and 8:00 a.m. Blood samples were taken after at least three weeks of treatment. Sampling was performed between 9:00 and 10 a.m., using evacuated tubes containing EDTA. Blood was then centrifuged, plasma was transferred to polypropylene tubes and kept frozen at - 20° C until analysis.

Solutions and Sample Preparation

Solutions of stock reference standards of racemic FLU, racemic N-FLU, (R)-N-FLU and clozapine (1 mg/mL, 10 μg/mL and 1 ug/mL) were prepared in methanol and stored below 0°C. Dilutions were made fresh daily for each analysis. Plasma standards were prepared daily by adding known amounts of the stock standards to blank human plasma.

A 1.5 mL aliquot of plasma, with 150 μl of clozapine as internal standard (200 ng/mL methanolic solution) and 500 μl 1N NaOH added, was vortex-shaken for 30 sec and transferred to an Extrelut-3 glass column. After 10 min, the analytes were eluted under gravity with 5 mL n-hexane-isoamyl alcohol (97:3). The organic phase was evaporated to dryness under a stream of nitrogen and redissolved in 150 μl of HPLC mobile phase. A 100 μl volume was injected into the HPLC column.

Calibration, Analytical Recovery and Precision

Spiked plasma carried through the entire procedure was used to create calibration curves and to determine analytical recoveries, intra-day and inter-day variabilites. The linearity of the calibration curves was studied in the range of 10-2000 ng/mL for each analyte.

Analytical recoveries were perfomed at three different concentrations (10, 100, and 500 ng/mL for each substance) with 5 samples for each concentration. The same concentrations were used to test the analytical imprecision, performing analyses of serum samples for up to six days.

Drugs Interferences

Several drugs commonly administered to individuals with depressive disorders were examined for their possible interference with the determination of fluoxetine and its metabolite stereoisomers. The substances tested were: imipramine, amitriptyline and their active metabolites desipramine and nortriptyline.

One microgram of each drug was added to blank plasma and to plasma spiked with FLU and N-FLU enantiomers carried through extraction procedure and analyzed by HPLC.

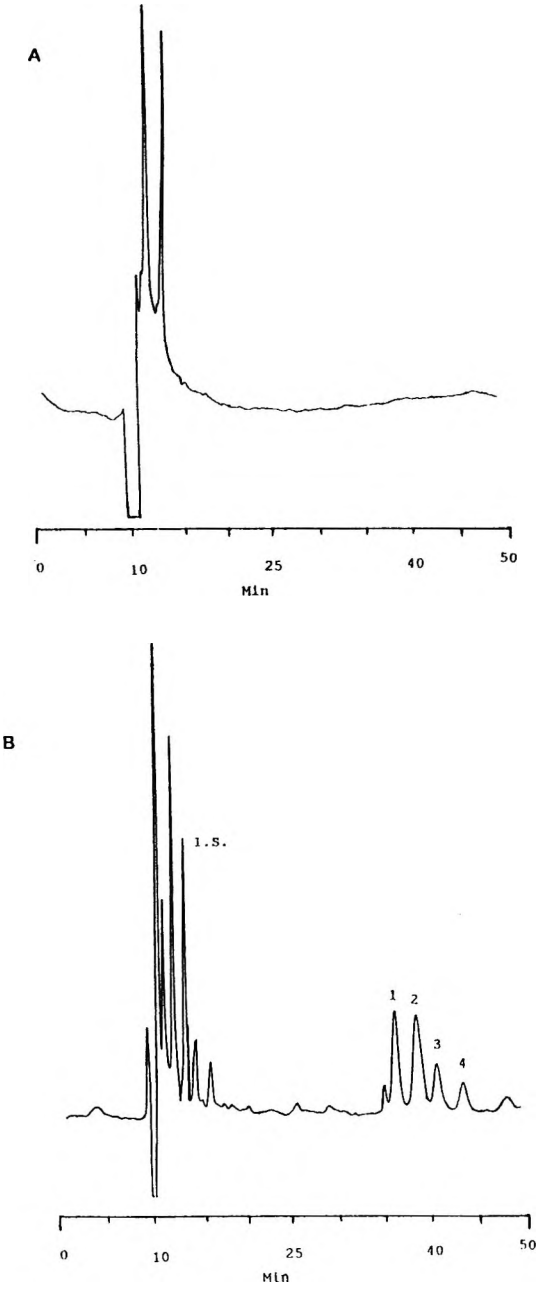

Figure 2. Chromatogram of (A) blank plasma; (B) an extract of a plasma sample containing 148 ng/mL (S)-N-FLU (1), 147 ng/mL (R)-N-FLU (2), 52 ng/mL (S)-FLU (3), 39 ng/mL (R)-FLU (4) and 200 ng/mL clozapine (I.S.).

Table 1

Recovery and Variability (n=5)

Concentration (ng/mL)	Recovery (mean + S.D.) (%)	Variability (%)	
		Intraday	Interday
(R)-FLU			
10	90.9 ± 3.4	3.7	3.9
100	91.2 ± 3.4	3.7	3.9
500	91.5 ± 3.6	3.9	4.1
(S)-FLU			
10	94.0 ± 3.4	3.6	3.8
100	94.1 ± 3.4	3.6	3.8
500	94.4 ± 3.5	3.7	4.0
(R)-N-FLU			
10	84.0 ± 2.3	2.7	2.9
100	84.3 ± 2.3	2.7	3.0
500	84.7 ± 2.4	2.8	3.1
(S)-N-FLU			
10	81.9 ± 2.1	2.5	2.9
100	82.3 ± 2.3	2.8	3.0
500	82.9 ± 2.4	2.9	3.1

RESULTS AND DISCUSSION

Fig. 2 depicts representative chromatograms of extracts of blank plasma sample and plasma sample containing both the enantiomers of FLU and N-FLU. Under the conditions outlined here, the retention times of (S)-N-FLU and (R)-N-FLU were 37 and 40 minutes while those of (S)-FLU and (R)-FLU were 41.6 and 44.6 minutes.

Extract of blank plasma showed that no chromatographic peak interfered with the analysis of enantiomers of FLU and N-FLU and with the internal standard clozapine, whose ritention time was 13 minutes.

The chiral separation was achieved only using an on line-coupled two column system, with the second column heated at 40°C for the improvement of peak width and shape.

Table 2

Detection Limit and Liability

Compound	Retention time (min)	Detection limit (ng/mL)	Linearity
(S)-Fluoxetine	41.6	10	y = 3.7x + 0.7
(R)-Fluoxetine	44.6	10	y = 3.4x + 0.7
(S)-Norfluoxetine	37.0	10	y = 5.6x + 1.3
(R)-Norfluoxetine	40.0	10	y = 5.2x + 1.2

y = peak height (cm)
x =m amount of the analytes (μg/mL)

Table 3

Patient Plasma Concentration of (S)-FLU, (R)-FLU (S)-N-FLU, (R)-N-FLU

Patient	Plasma Concentration (ng/mL)				Ratio	
	(S)-FLU	(R)-FLU	(S)-N-FLU	(R)-N-FLU	(S)/(R)FLU	(S)/(R)N-FLU
1	100	100	156	123	1.01	1.26
2	38	33	68	37	1.15	1.83
3	40	33	42	38	1.21	1.10
4	67	50	78	46	1.34	1.69
5	62	37	74	161	1.68	0.35
6	121	85	101	73	1.42	1.37
7	112	66	125	113	1.71	1.10
8	60	37	50	64	1.63	0.78
9	128	114	91	80	1.12	1.13
10	96	83	84	147	1.16	0.56
Mean	82.4	63.8	86.9	88.2	1.34	1.12
S.D.	33.1	30.1	34.0	45.2	0.26	0.46

S.D. = Standard Deviation

None of the other antidepressant drugs tested interfered with the assay.

The analytical recoveries of all the analytes, and the intra-day and inter-day variabilities are shown in Table 1.

The detection limit (signal-to-noise ratio of 3), the linearity of the method and the ritention times of the peaks are shown in Table 2. The calibration curves were linear over the range 10-2000 ng/mL for both the enantiomers of FLU and N-FLU with correlation coefficients always higher than 0.99.

This method has been applied in our laboratory to monitor the concentrations of enantiomers of fluoxetine and its metabolites in ten patients treated with 20 mg/die of the drug. Table 3 lists the data obtained, which are comparable with those reported in the literature.[15,16] Since plasma levels of (S)- and (R)- enantiomers of FLU and N-FLU were enough different, a stereospecific metabolism is confirmed.

In summary, the HPLC method described here permits quick and simple extraction and simultaneous determination of both the enantiomers of fluoxetine and norfluoxetine, without any derivatization and interference from two first-generation tricyclics. The development of this enantiospecific assay could be of great help in future studies on stereospecific pharmacokinetics and pharmacodynamics of fluoxetine, which can improve its clinical use.[17]

ACKNOWLEDGEMENTS

We thank Mrs. Simonetta Di Carlo and Mrs. Antonella Bacosi for expert technical assistance.

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Received November 24, 1995
Accepted December 15, 1995
Manuscript 4034

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1937–1945 (1996)

MIXED MODE S. P. E. OF POLLUTANTS IN WATER ON MECHANICALLY BLENDED SILICA-BASED BONDED PHASES. I. INFLUENCE OF THE PREPARATION METHOD

Z. Hajouj[1], J. Thomas,*[1] A. M. Siouffi[2]

[1] Laboratoire de Chimie
Faculté des Sciences et Techniques
Parc de Grandmont
37200 Tours - France

[2] Laboratoire de Génie Chimique et Chimie Appliquée
Faculté des Sciences et Techniques Saint-Jérôme
Avenue Escadrille Normandie-Niémen
13397 Marseille - France

ABSTRACT

Cartridges for Solid-Phase Extraction operating in mixed mode were filled with two phases: C_{18} bonded silica and propylsulfonic acid bonded silica, mechanically blended according to two methods: one dry, the other by suspension in acetonitrile. They were compared with cartridges containing the individual phases, and with a mixed phase having two functionalities bonded onto a common silica particle. The percent recoveries of neutral, basic and acidic environmental pollutants and their reproducibilities were determined by HPLC. After

evaluation of the optimum eluent volume, the blended phase prepared without solvent and very rapidly, was overall very promising in terms of retention, reproducibility and composition potential.

INTRODUCTION

In Solid-Phase Extraction (S.P.E.) applied to environmental analysis, bonded phases operating with double mechanism have recently been introduced with the aim of maximum retention of the different classes of pollutants on the cartridges. There is a category of mixed phases with two functionalities bonded onto the same particle.[1-3] Variants are represented respectively by a functional group bonded in low concentration onto a resin,[4] and an impurity included in the polymer network.[5]

There is also the category of separate bonded phases whose particles are blended mechanically, and which have only been the subject of very limited and recent work.[3,6] The arrangement of phases in tandem[7-10] could be regarded as a variant.

The potential of this second category rests in its very high flexibility for a given sample. It is, in fact, possible to combine functional groups suitable both in interaction types and proportions. A requirement for this line of research, is to verify that the level of variation of the percent recoveries is acceptable.

Since no information had been published in this area, we have examined two practical methods of cartridge filling. Using an aqueous sample of neutral, acidic and basic environmental pollutants, the results obtained were compared with extraction carried out on individual phases, and on a commercial mixed phase.

MATERIALS

Chemical and Reagents

The solvents (Carlo Erba, Milan, Italy) and the water, purified by the Milli-Q system (Millipore, Bedford, MA, U.S.A.), were all of HPLC quality. The ammonium hydroxide in 28 % solution (Carlo Erba) and diammonium hydrogen phosphate (Merck, Darmstadt, Germany) were of analytical quality.

The p-cresol, anthracene, phenanthrene and fluoranthene (Aldrich, Steinheim, Germany) were 98 % pure. The acridine, phenothiazine, phenazine (Sigma, St. Louis, MO, U.S.A.) and quinoline (Janssen Chimica, Geel, Belgium) were 99 % pure.

A stock solution of a mixture of these compounds was prepared in acetonitrile at a concentration of 1.125 $g.L^{-1}$ total matter, then stored in a refrigerator at 4°C.

Sample solution : a 0.064 mL sample of the stock solution was mixed with acetonitrile up to a volume of 7.5 mL, to which was added 42.5 mL of water. The resulting sample solution was 15 % in acetonitrile, allowing complete dissolution of the P.A.H.

The glass extraction columns (J.T. Baker, Phillipsburg, NJ, U.S.A.) were of 8 mL volume. The polyethylene frits (J.T. Baker) were 12 mm in diameter with 20 µm porosity.

The phases used for the extraction had groups bonded onto silica gel. The Baker Bond octadecyl (C_{18}) phase (J.T. Baker) had the following properties: d_p = 40 µm, pore diameter = 60 Å, bonded carbon = 17 %, specific surface area = 500 $m^2.g^{-1}$.

The Bondesil PRS propylsulfonic acid phase (Analytichem International, Harbor City, CA, U.S.A.) had the following properties: d_p = 40 µm, pore diameter = 60 Å, exchange capacity = 0.18 meq. g^{-1}, specific surface area = 350 $m^2.g^{-1}$.

The characteristics of the commercial mixed phase Bond Elut Certify were not available.

Equipment

The extractions were carried out with a Visiprep Vacuum Manifold (Supelco, Inc., Bellefonte, PA, U.S.A.).

The HPLC system was composed of a model 114 M pump (Beckman, San Ramon, CA, U.S.A.), a model C6W injector (Valco, Houston, TX, U.S.A.), a model 655A variable wavelength U.V. detector (Merck) fitted with an 11 µl cell, and a Chromjet model integrator-calculator (Spectra-Physics, San Jose, CA, U.S.A.).

METHODS

Cartridge Preparation

An equal quantity of each phase, slightly more than 100 mg, was introduced into a test tube containing a magnetic stirrer.

Method A

The homogeneity was achieved, without solvent, by vigorous magnetic stirring for 2 minutes. After inserting a frit, 100 mg of the mixture was introduced into the cartridge where a second frit retained the phase.

Method B

After addition of 2 mL of acetonitrile, the homogeneity was achieved by vigorous magnetic stirring for 5 minutes. The suspension was transferred into a cartridge containing a frit. The phase was dried under vacuum for six hours with a water aspirator pump. After weighing, additions of suspension interspersed with the drying periods enabled the quantity of the mixed phase to be adjusted to the desired level. The mixed phase was retained with a second frit.

Extraction

Only a single cartridge was used for each operation of the Visiprep apparatus. For each operation, the flow rate was 1 $mL.min^{-1}$ considering the slow ion exchange kinetics.

Conditioning was carried out by successive introductions of 2 mL of methanol and 2 mL of water. After attachment of a 50 mL reservoir onto the cartridge, the sample solution was applied to it. The reservoir was washed with 2 mL of water, then the cartridge was connected to a water aspirator pump for drying for 15 minutes.

After elution with 8 mL of acetonitrile, 2 % in NH_4OH, the extract was collected in a graduated flask, neutralized with 3 drops of concentrated HCl, then the level was adjusted to 20 mL with 2 mL of acetonitrile and 10 mL of aqueous buffer (pH = 7.0). The solution was filtered on a Nylon Acrodisc 13 (Gelman Sciences, Ann Arbor, MI, U.S.A.) of porosity 0.2 μm, the only filter not releasing substances interfering with the compounds analyzed.

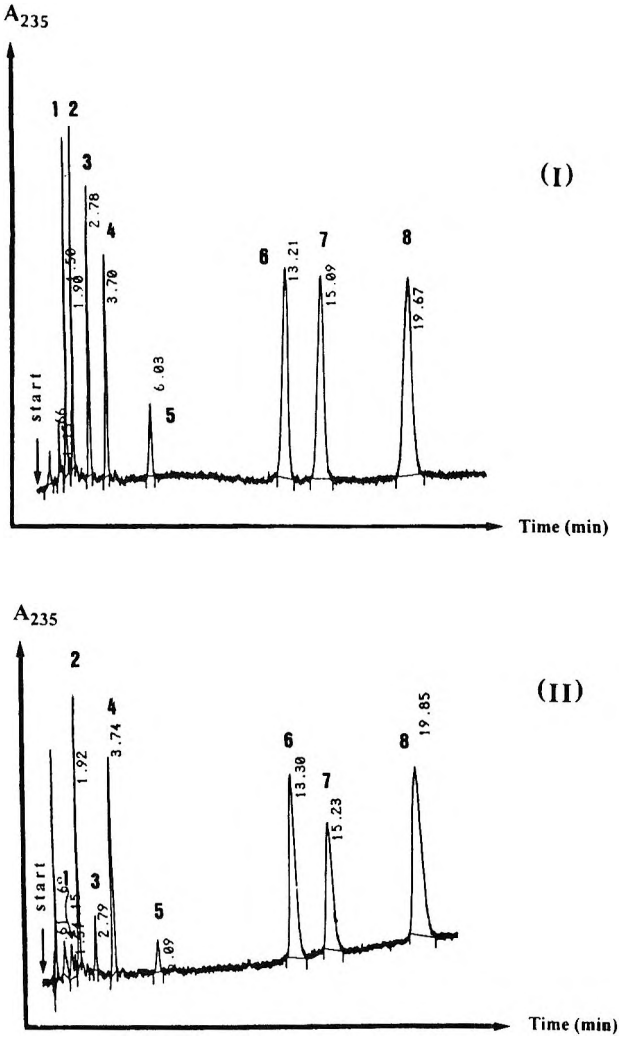

Figure 1. Typical chromatograms of a reference solution (I) and a solution obtained after S.P.E. on a cartridge prepared by mixing the phases without solvent (II). Conditions : column : 125 x 4 mm ; stationary phase : ODS 2 Spherisorb, d_p = 5 µm ; mobile phase : acetonitrile / 0.05 M $(NH_4)_2$ H PO_4 buffer (pH = 7.0) 50/50, flow-rate : 2 $mL.min^{-1}$; injection volume : 20 µl ; U.V. detection : 235 nm ; temperature : 20 ± 2°C ; compounds : 1 = p-cresol, 2 = quinoline, 3 = phenazine, 4 = acridine, 5 = phenothiazine, 6 = fluoranthene, 7 = anthracene, 8 = phenanthrene.

Quantitation

Reference solution: a 0.064 mL sample of the stock solution was diluted to 10 mL with acetonitrile, mixed with 10 mL of aqueous buffer (pH = 7.0), then filtered.

Each quantitative determination was performed by comparison with a reference solution, freshly prepared and injected before the extracted sample solution. Two typical chromatograms are shown (Fig. 1).

RESULTS AND DISCUSSION

Although an adsorbent having an organic polymer as base offers better retention of neutral and basic substances than a silica-based adsorbent in mixed mode,[1] we chose to study 50/50 mass percent mechanical mixtures of phases bonded onto silica: an octadecyl phase, C_{18}, and a propylsulfonic phase, PRS. This did not prevent comparison with a commercial mixed phase, Bond Elut Certify,[3] in which these two functions were bonded onto the same silica particle. Above all, this choice could lead in the future to the use of the greatest number of individual functions.

A mixture of neutral compounds: anthracene, phenanthrene, fluoranthene, and basic compounds with one or two nitrogen atoms : quinoline, acridine, phenazine, phenothiazine was suitable for these mixed phases. The acidic compounds were not retained by a cation exchanger. To represent this group, we therefore chose para-cresol which is a very weak acid with hydrophobic properties.

Several parameters affect the percent recovery: the sample solution volume, the type of sample solvent, the amount of matter in the sample, the flow rate during sample application, the quantity of adsorbent, the composition of the phase mixture, the type of elution solvent, the volume of eluent and the flow rate of the eluent.

A preliminary study was carried out on the quantity of adsorbent. This led us to use a mass of 100 mg. With fixed values for the other parameters (Table 1), this mass was sufficient to reach or exceed the cartridge capacity, the breakthrough level, depending on the compounds studies. This allowed better comparison of the differences in retention of these substances.

Table 1

Percentage Recoveries and R.S.D. (%) of Pollutants*, Using Different Elution Volumes, Different Phases and Two Different Mechanical Mixing Preparation Methods, All Other Parameters Being Fixed**

Phase Type	Bond Elut Certify			----PRS/C_{18} Mechanical---- Mixture 50/50 W/W			C_{18}	PRS
Prepration Mode				----------A----------			B	
Compound Elution Volume (mL)	4	6	8	4	6	8	8	8	8
p-cresol	traces	traces	traces	traces	traces	traces	traces	traces	
quinoline	95 ± 6	95 ± 6	98 ± 3	81 ± 8	101 ± 7	98 ± 4	111 ± 4	30 ± 6	101 ± 5
phenazine	65 ± 11	53 ± 7	58 ± 16	14 ± 4	14 ± 5	14 ± 4	16 ± 12	33 ± 9	
acridine	99 ± 7	100 ± 6	97 ± 4	85 ± 6	103 ± 10	101 ± 4	96 ± 2	99 ± 4	109 ± 4
phenothiazine	72 ± 19	73 ± 2	74 ± 13	62 ± 3	63 ± 6	66 ± 8	90 ± 10	101 ± 7	
fluoranthene	98 ± 10	96 ± 2	92 ± 2	90 ± 7	87 ± 15	86 ± 10	90 ± 2	103 ± 1	4 ± 6
anthracene	43 ± 19	79 ± 5	82 ± 1	56 ± 14	83 ± 10	81 ± 4	63 ± 4	76 ± 5	14 ± 5
phenanthrene	94 ± 8	91 ± 4	91 ± 2	76 ± 13	88 ± 15	93 ± 2	98 ± 2	95 ± 7	5 ± 5

* Each result is the mean of 4 extractions, each extract having been injected 3 times.

**Solid phase: total weight: 100 mg; sample: total weight: 0.072 mg, solution volume: 50 mL of deionized water/acetonitrile (85/15 v/v), flow rate: 1 $ml.min^{-1}$; elution: solvent: acetonitrile with 2 % ammonium hydroxide, flow rate: 1 $mL.min^{-1}$.

In addition, it was necessary to verify the complete recovery of the retained matter. We, thus, studied the effect of the variation, from 4 to 8 mL, of the eluent volume on the percent recovery and the relative standard deviations (R.S.D.). This effect was studied on two adsorbents: one double bonded, and the other a mechanical mixture of phases prepared without solvent. It was observed (Table 1) that a volume of 4 mL was not sufficient to desorb the anthracene, whatever the phase type.

The same was the case, to a lesser extent, for quinoline and phenanthrene extracted on the mechanically blended phase . Larger volumes, 6 or 8 mL, led to comparable percent recoveries, whatever the phase. It was also observed that the R.S.D. diminished most frequently with an increase in eluent volume. The volume of 8 mL was, thus, selected for the subsequent studies.

We could now compare a mechanical mixture prepared without solvent (method A), with the same mixture in acetonitrile suspension (method B), these

two preparations having potentially very different homogeneities. The comparison was extended to the individual C_{18} and PRS phases (Table 1). As regards percent recoveries, it was the mixture prepared without solvent, which showed the behavior closest to that of the commercial mixed phase, despite a lower ability to extract phenazine. The individual C_{18} and PRS phases showed their weak retention power to the least hydrophobic basic compounds, and the most hydrophobic compounds, respectively. None of the phases retained the acidic compound.

As for the reproducibilities, the range between the smallest and largest R.S.D. values (%) is considered for each phase: 1-9 for the C_{18} phase, 1-16 for the Bond Elut Certify phase, 2-10 for the mechanical mixture prepared by method A and 2-12 for that prepared by method B. The blended phase prepared dry, thus, showed a R.S.D. range very similar to that of an individual homogenous C_{18} phase, and narrower than that obtained with the double-bonded phase.

CONCLUSION

From the performance point of view, and considering its very rapid preparation, the dry-blended phase seems competitive with the double-bonded commercial phase. This justifies further studies considering the great flexibility of composition available.

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Received September 11, 1995
Accepted December 7, 1995
Manuscript 3928

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1947–1956 (1996)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ISOXAZOLYL-NAPHTHOQUINONES: A COMPARISON BETWEEN EXPERIMENTAL AND THEORETICAL LIPOPHILICITY

Marcela Longhi, Marcela Linares, María M. de Bertorello*

Departamento de Farmacia
Facultad de Ciencias Químicas
Universidad Nacional de Córdoba
Sucursal 16, Casilla de Correo 61
5016-Córdoba, Argentina

ABSTRACT

An RP-HPLC procedure was developed for determining the lipophilicity of a series of isoxazolyl-naphthoquinones which possess antibacterial, trypanosidal and antineoplasic activity. The experimental results were compared with theoretical log P values, and it was found that there was a good relationship between the two methods, except for very lipophilic compounds.

INTRODUCTION

In search of bioactive compounds, we prepared a series of naphthoquinones bearing different isoxazole substituents.[1-3] Extensive studies carried out with some of these compounds have revealed antibacterial,[4,5] trypanosidal[6] and antineoplasic[7] activity.

The lipophilicity of drugs has been shown repeatedly,[8-10] to be of great importance in determining the body distribution, as well as the relative potency of drugs that are members of an analogous series. A useful descriptor of global lipophilicity has been the octanol-water partition coefficient (log P_{oct}), traditionally obtained by the shake-flask method.

Because this method has a number of disadvantages, other procedures have been developed, for example chromatographic, such as reverse phase high performance liquid chromatography (RP-HPLC).[11-12] This method assumes a linear relationship between the logarithm of capacity factor (log k´) and log P, by a Collander-type equation.[13]

The reason for log P being accurately determined by RP-HPLC is that the dominant mode of retention in the stationary phase is that of partitioning, not absorption.[14]

In addition to the experimental methods, theoretical procedures for the calculation of log P values have been developed.[8,15]

The aim of this study was to determine the lipophilicity of a series of isoxazolyl-naphthoquinones, because between their members are biologically relevant molecules, and the knowledge of this parameter is important in view of their possible clinical use. We selected the following compounds:

1a-2-(3,4-dimethyl-5-isoxazolylamine)-N-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone- 4-imine.

1b- 2-(4-methyl-5-isoxazolylamine)-N-(4-methyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine.

1c- 2-(5-methyl-3-isoxazolylamine)-N-(5-methyl-3-isoxazolyl)-1,4-naphthoquinone-4-imine.

2a- 4-N-(3,4-dimethyl-5-isoxazolyl)-1,2-naphthoquinone.

2b- 4-N-(4-methyl-5-isoxazolyl)-1,2-naphthoquinone.

2c- 4-N-(5-methyl-3-isoxazolyl)-1,2-naphthoquinone.

3a- 2-hydroxy-N-(3,4-dimethyl-5-isoxazoyl)-1,4-naphthoquinone-4-imine.

3b- 2-hydroxy-N-(4-methyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine.

3c- 2-hydroxy-N-(5-methyl-3-isoxazolyl)-1,4-naphthoquinone-4-imine.

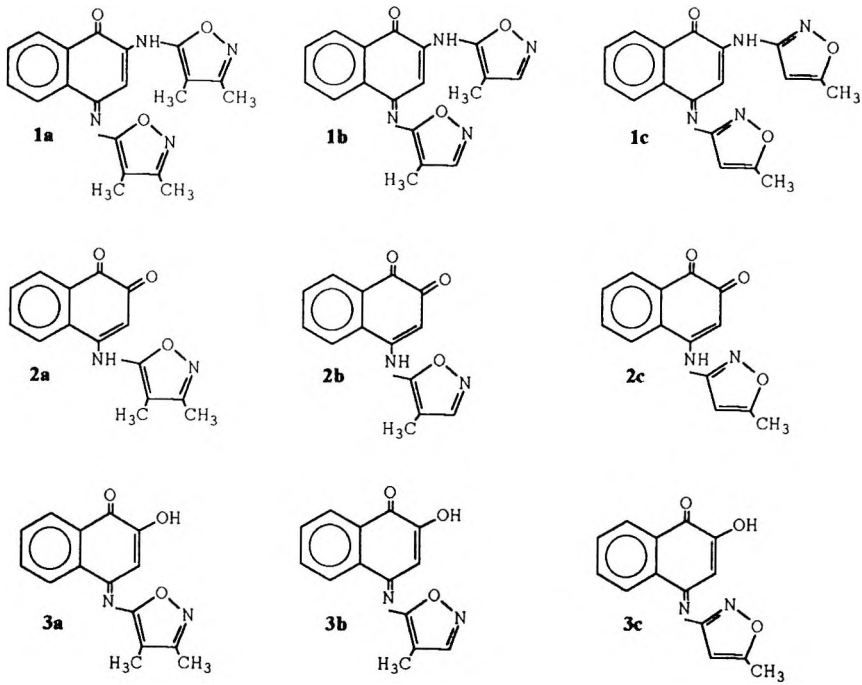

Figure 1. Chemical structures of compounds studied.

We chose the RP-HPLC technique due to the low water solubility of these compounds. The reliability of this methodology is checked by comparison of the experimental data with the calculated log P values.

MATERIALS AND METHODS

Materials

The isoxazolyl-naphthoquinone derivatives (1-3) were obtained as in previously reported procedures.[1-3] All other chemicals and solvents were of analytical reagent grade and were used without further purification. Reagent grade water was generated by a Millipore Milli-Q Water purification system.

Chromatography

HPLC chromatography was performed with a KONIK model 500G, with a UV-V-KNK-029-757 absorbance detector with the wavelength set at 245 nm, a Rheodyne 7125 injector, a Spectra Physics 4600 Data Jet integrator, and a 250 x 4.6 mm Supelcosil LC-18 5-μm HPLC column (Supelco). The mobile phase composition ranged from 60 to 90% (v/v) methanol with water. The flow rate was 1.0 mL/min.

Analytes were dissolved in methanol and then they were injected separately from each other. The experiments were repeated three times and the mean value of the retention time for each compound was determined.

Retention times (t_r) can be transformed into a capacity factor as $k' = (t_r - t_0)/t_0$ where t_r and t_0 are the retention times of the analytes and the methanol, respectively. Capacity factors (log k′) were determined at six to seven different concentrations of methanol in water (90%, 85%, 80%, 75%, 70%, 65%, and 60%). Experiments with lower percentage of methanol than 60%, afforded retention times too long to be measured.

The average log k′was graphed against the percent of methanol, and the value of log k_w (where k_w represents the capacity factor in absence of organic solvent) was obtained by extrapolating to 100% water, according to the following equation: $\log k' = ax + \log k_w$. The extrapolated log k_w values are used in order to suppress the effect of the organic modifier and to obtain lipophilicity values independent of the eluent conditions. The system was calibrated by determining log k_w for a set of compounds, which included the following ones: pyridine (log P = 0.64), aniline (log P = 1.08), acetanilide (log P = 1.42), 1,4-naphthoquinone (log P = 1.71), p-nitroacetanilide (log P = 2.34), 1-naphthol (log P = 2.98), and phenanthrene (log P = 4.46).

Log P Calculations

For the calculation of log P of all studied isoxazolyl-naphthoquinones we used the Leo-Hansch fragmental method.[8]

RESULTS AND DISCUSSION

The chemical formulae of the tested isoxazolyl-naphthoquinones are given in Figure 1.

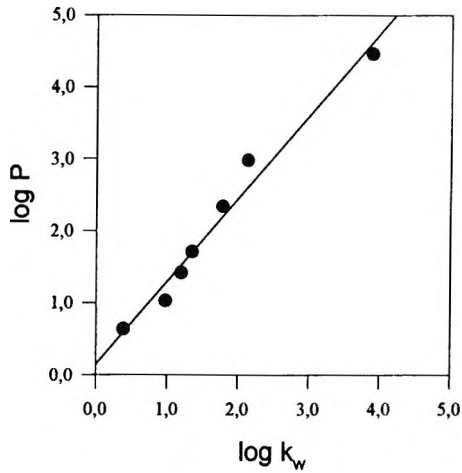

Figure 2. Relationships between log P and log k_w for selected standards.

Determination of Partition Coefficients by HPLC

The application of an HPLC system for the determination of partition coefficients by correlation, requires previous calibration of the system using standards for which classical shake-flask partition coefficients are known.[16] In our case, we selected seven compounds, which exhibited intense UV absorption at 245 nm, and the set of standards chosen covered a log P range from 0.64 (pyridine) to 4.46 (phenanthrene), where most of the log P values for the naphthoquinone derivatives could be included.

As shown in Figure 2, excellent correlations were obtained for all standards assayed, and the relationship between log k_w and log P for the set of standards was fitted into the following linear equation:

$$\log P = 1.15\ (\pm 0.08)\ \log kw + 0.15\ (\pm 0.16) \qquad (1)$$

with n = 7, $r^2 = 0.992$, and S = 0.051, where n is the number of data used, r^2 the correlation coefficient, S the estimated standard error, and the 95% confidence limits on the regression coefficients are given in parenthesis. This correlation can be considered as very satisfactory.

To estimate reproducibility of retention times and, consequently, of log k′ parameters, the above standards were tested. As depicted in Table 1, the results showed excellent reproducibility, which, allowed us to perform the whole HPLC analysis with three independent injection runs for every solute.

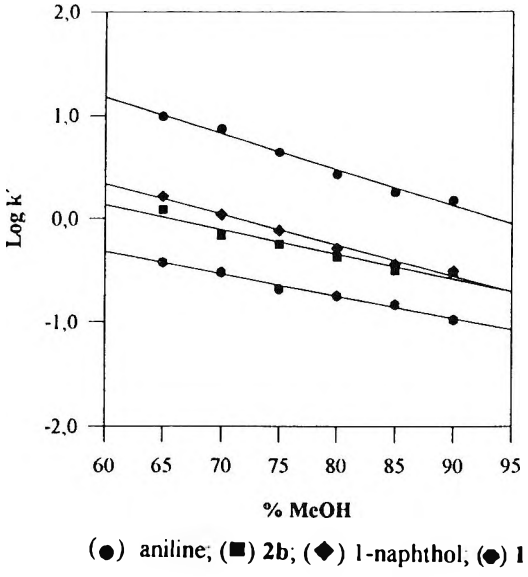

Figure 3. Typical graph of log k´at different methanol concentrations.

Table 1

Dispersion Analysis for Retention Times (R_t) and log k' Values of Seven Calibration Standards. Mobile Phase: Methanol-Water 75:25 (v/v)

Standards	n	Rt (min) ± SD	log k'
Pyridine	7	3.01 ± 0.02	-0.755
Aniline	7	3.10 ± 0.03	-0.676
Acetanilide	7	3.28 ± 0.05	-0.551
1 Naphthoquinone	7	3.38 ± 0.03	-0.494
p-Nitroacetanilide	8	4.30 ± 0.05	-0.168
1-Naphthol	8	4.54 ± 0.04	-0.112
Phenanthrene	7	5.88 ± 0.06	0.113

Table 2

Experimental and Calculated Lipophilicity Values

Compound	log kw	log P (RP-HPLC)	log P (CLOGP)
1a	3/93	4.68	4.50
1b	3.34	4.00	4.12
1c	3.87	4.61	4.26
2a	2.36	2.87	3.01
2b	1.74	2.15	2.37
2c	2.96	3.56	3.33
3a	2.79	3.37	3.51
3b	2.72	3.28	3.44
3c	6.32	7.44	5.86

The above HPLC analytical treatment was then applied to compounds 1-3. Thus, respective log k′ values were obtained from analysis of the retention behaviour, using the same methanol volume fractions as in standards. The log k′ of the compounds and standards, decreased linearly with increasing methanol percentage of mobile phase (Figure 3).

The log k_w values for each naphthoquinone analogue, were obtained by regression analysis of log k′ data. Then, extrapolation of respective log k_w values in equation 1, permitted calculation of the corresponding partition coefficients of the derivatives assayed. These results are depicted in Table 2.

Calculated Log P

The log P values for the nine naphthoquinones were calculated by means of the fragmental method (CLOGP) of Leo and Hansch, which is based on the additivity of fragmental contributions. These results are shown in Table 2. When it was neccesary, appropriate correction factors were applied.[15]

Correlation Between Lipophilic Indexes

The lipophilicity values of the naphthoquinones determined by HPLC, have been compared to calculate log P values (Figure 4). A close relationship has been found to exist between these pairs of values, according to the equation ($n = 9$, $r^2 = 0.962$, $S = 0.084$):

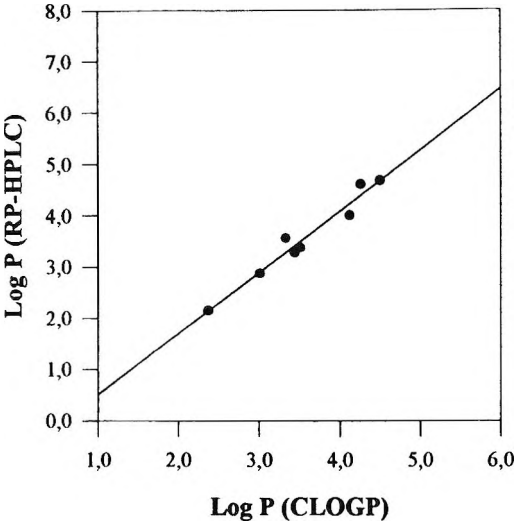

Figure 4. Correlation between calculated and experimental log P values.

$$\log P\ (\text{RP-HPLC}) = 1.19\ (\pm 0.11)\ \log P\ (\text{CLOGP}) - 0.68\ (\pm 0.44) \quad (2)$$

The plot indicates a linear relationship between the experimental and calculated values, with a slope closer to unity, that allows us to postulate that lipophilicity of new isoxazolyl-naphthoquinone analogues could be predicted from their retention in HPLC using Eq. 2. However, in Fig. 4 we can see that the data point of 3c appreciably deviated from linearity and exhibited a lipophilicity much higher than other analogues.

Using the RP-HPLC technique, we observed a log P value of 7.44 for 3c, which lies above the upper limit of accuracy (log P = 4.60) for most experimental methods for measuring log P. For this reason, the log P values of very lipophilic molecules are calculated, rather than measured.[17] This latter fact obviously indicates the limit of the applicability of Eq. 2 for very lipophilic compounds.

On the other hand, it may be interesting to compare the log P values of the keto/enol tautomers of the three series: a, b, and c. In all cases, it was observed that the enol forms, as was established in other works for related compounds,[15] are always more lipophilic and, in addition, 3c has the highest lipophilicity in the group of compounds studied.

CONCLUSIONS

We conclude, that with some few exceptions (very lipophilic compounds), the log P values of the isoxazolyl-naphthoquinone derivatives in n-octanol/water can be determined using RP-HPLC, and that the log P of all these compounds can be calculated from the theoretical method of Leo and Hansch.

ACKNOWLEDGEMENTS

The authors thank CONICET, the Secretaría de Ciencia y Técnica de la Universidad Nacional de Córdoba, and the Consejo de Investigaciones Científicas y Tecnológicas de la Provincia de Córdoba (CONICOR), for financial support.

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Received December 15, 1995
Accepted January 3, 1996
Manuscript 4051

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1957–1966 (1996)

DETERMINATION OF THEOPHYLLINE IN PHARMACEUTICALS BY MICELLAR LIQUID CHROMATOGRAPHY AND SPECTROPHOTOMETRIC DETECTION

I. Perez-Martinez, S. Sagrado,
M. J. Medina-Hernández*

Departamento de Química Analítica
Facultad de Farmacia
Universidad de Valencia
C/ Vicente A. Estellés s/n
E-46100 Burjassot, Valencia, Spain

ABSTRACT

An HPLC procedure for the determination of theophylline in pharmaceutical preparations is described. A Spherisorb octadecylsilane ODS-2 C_{18} analytical column and spectrophotometric detection at 273 nm were used. Adequate retention was achieved with a mobile phase containing 0.05 M sodium dodecylsulphate (SDS) and 3% propanol at pH 7. The reproducibilities were 1.2 % and 1.7 % for 3.8 and 7.6 μg/mL theophylline concentrations, respectively.

The determination of theophylline in six pharmaceutical preparations gave recoveries, with respect to the values declared by the manufacturers, which usually ranged between 83-97 % and 85-104% using peak heights and peak areas, respectively.

INTRODUCTION

Theophylline (1,3-dimethylxanthine), is a bronchodilator agent mainly used in the treatment of chronic asthma, bronchitis, emphysema and apnea in newborn children. It has a narrow therapeutic range and serum-theophyllineconcentrations should be monitored during therapy.

Adverse effects commonly affect the gastro-intestinal tract and central nervous system. Following overdosage tremor, delirium, convulsions and death may occur.[1]

Several analytical techniques have been applied to the determination of theophylline in pharmaceuticals, spectrophotometry,[2] phosphorimetry,[3] gas chromatography[4] and capillary electrophoresis.[5] However, high performance liquid chromatography (HPLC) now seems to be the most frequently used technique.

In these procedures, a C_{18} stationary phase, a mixture of acetonitrile-wateror methanol-water with acetate and phosphate buffers as mobile phases and UV detection was usually used.[6-7]

Micellar liquid chromatography (MLC) is an alternative of reversed phase liquid chromatography, which employs aqueous solutions of surfactants above the micellar critical concentration as the mobile phases. Procedures for the evaluation of diuretics,[8,9] anabolic steroids,[10] and catecholamines[11] in pharmaceuticals, have been developed.

The main advantages to using a micellar solution, instead of a conventional hydroorganic mobile phase, in reversed phase liquid chromatography, are the lower cost and toxicity, the biodegradability of the solvent, the performance of elution gradients of surfactant without the need of reequilibration of the column,[12] and the easy solubilization of analytical samples, which allows the determination of drugs in physiological fluids without the need of a previous separation of the proteins present in the samples.[13]

In a previous paper, a micellar liquid chromatographic procedure for the determination of caffeine, theophylline and theobromine in urine samples was described.[14] Maximum resolution was achieved with a 0.075 M sodium dodecylsulphate + 1.5% propanol eluent.

In this paper, investigations on the chromatographic behaviour of theophylline with micellar eluents, are reported and a rapid analytical procedure for the determination of this compound in pharmaceutical formulations is developed.

MATERIALS AND METHODS

Apparatus

A Hewlett-Packard HP 1050 chromatograph with a quaternary pump, a UV-visible detector and an HP 3396A integrator was used (Palo Alto, CA, USA). Data acquisition was made with the Peak-96 software from Hewlett-Packard (Avondale, PA, USA). The solutions were injected into the chromatograph through a Rheodyne valve (Cotati, CA, USA) with a 20 μL loop. A Spherisorb octadecylsilane ODS-2 C_{18} column (5 μm, 120 x 4.6 mm) and a guard column of similar characteristics (35 x 4.6 mm) (Scharlau, Barcelona, Spain) were used. The mobile phase flow rate was 1 mL min^{-1}. The detection was performed in UV at 273 nm. All the assays were carried out at room temperature.

Reagents and standards

The micellar mobile phases were prepared by mixing aqueous solutions of sodium dodecylsulphate (99%, Merck, Darmstadt, Germany) with an alcohol to obtain the working concentration. The alcohols studied were methanol (HPLC, Panreac, Barcelona, Spain) and 1-propanol (analytical reagent, Panreac). The pH of the micellar eluent was adjusted with 0.01 M phosphate buffer, prepared with disodium hydrogen phosphate and phosphoric acid (analytical reagent, Panreac).

Stock standard solutions of theophylline (Fluka, Buchs, Switzerland, > 99%) were prepared by dissolving 10 mg of the compound in 100 mL of 0.05 M SDS solutions and they were stored in the dark at 4°C. Under such conditions, solutions were stable at least for one month. Working solutions were prepared by dilution of the stock standard solution.

Barnstead E-pure, deionized water (Sybron, Boston, MA, USA) was used throughout. The mobile phase and the solutions injected into the chromatograph, were vacuum-filtered through 0.45 μm and 0.22 μm Nylon membranes, respectively (Micron Separations, Westboro, MA, USA).

Sample preparation

For the analysis of tablets, five tablets were weighed and ground in a mortar. A portion was taken, weighed and dissolved in 0.05 M SDS in an ultrasonic bath. The solutions were filtered trough a n° 4 sintered glass plate and diluted in a calibrated flask. Capsules were dissolved in 0.05 M SDS, by immersion in an ultrasonic bath. An adequate volume of the drops was taken and diluted with 0.05 M SDS. Other dilutions were made with 0.05 M SDS. In all cases, triplicate or quintuplicate determinations were performed.

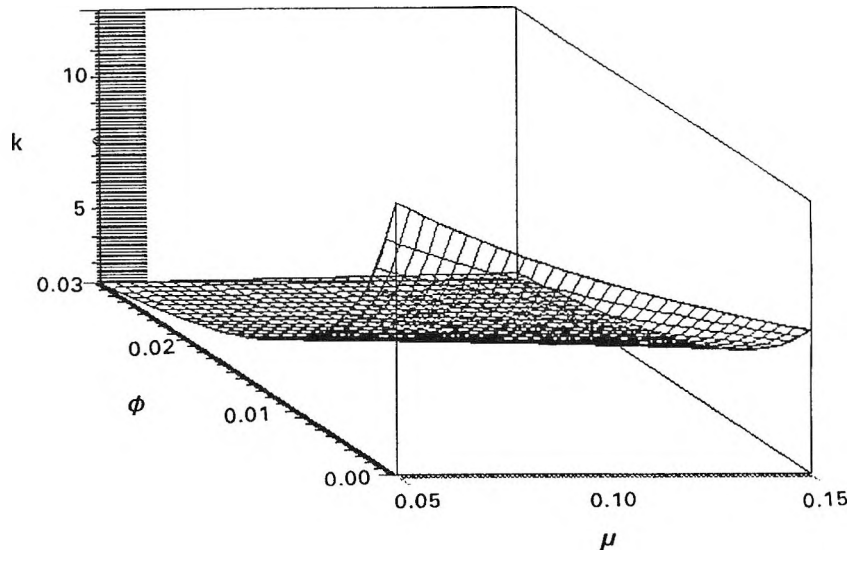

Figure 1. Retention surface for theophylline as a function of the concentration of surfactant, μ, and propanol, ϕ, in the mobile phase (pH = 7).

Table 1

Capacity Factors and Efficiency of Theophylline Obtained with Different SDS Mobile Phases

SDS, M	Modifier, v/v %	k	N
0.1	Methanol, 5%	4.0	66
	Propanol, 3%	2.0	147
0.05	None	12.6	27
	Propanol, 1.5%	4.0	144
	Propanol, 3%	2.2	475
0.1	Propanol, 1.5%	3.6	115
0.15	None	7.6	31
	Propanol, 1.5%	3.0	136
	Propanol, 3%	2.5	237

RESULTS

Chromatographic Behaviour of Theophylline

A study to select the composition of the mobile phase (pH, concentration of SDS, and nature and concentration of modifier), for the adequate retention of theophylline was performed.

No significant changes in the retention of theophylline were observed in the 3.5-6.9 pH range at a fixed 0.1 M SDS concentration, as can be expected owing the protonation constants of the compound in aqueous solutions ($\log K_1 = 8.6$ and $\log K_2 = 3.5$). Table 1 shows the capacity factors and efficiency values of the peaks of theophylline obtained with different mobile phases. The retention of theophylline decreased when the SDS concentration in the mobile phase increased. In a purely micellar medium, the peaks of theophylline, obtained for different SDS concentrations, were asymmetrical and the values of efficiency were very low and slightly modified with the mobile phase composition.

In MLC, the addition of an alcohol to the mobile phase produces, for most solutes, a decrease in retention and an improvement in the efficiency. A short-chain alcohol (methanol, 5% and propanol, 3%) was added to the 0.1 M SDS eluent. As can be observed, the addition of propanol to the 0.1 M SDS mobile phase produced adequate retention and improvement of the efficiency of the chromatographic peaks with respect to the use of methanol. As a consequence, propanol was selected.

In order to select the composition of the mobile phase (SDS and propanol concentrations), the equation of the retention of theophylline was obtained in agreement with the suggestions reported by Torres Lapasió et al.[15] The capacity factors of theophylline for the selected mobile phases (Table 1) were adjusted to an equation of the type:

$$\frac{1}{k'} = A\mu + B\phi + C\mu\phi + D \qquad (1)$$

where μ is the total concentration of surfactant, ϕ is the volume fraction of alcohol and A, B, C and D fitting parameters. The fitting parameters for theophylline calculated using multiple regression analysis were: 0.5204, 14.284, -35.588 and 5.3779×10^{-2}, respectively.

In this equation, the term $(B+C\mu)$ is a measure of the eluent strength of modifier in the presence of a constant concentration of the surfactant. On the other hand, the term $(A+C\phi)$ indicates the eluent strength of surfactant in the presence of a constant concentration of the modifier. High values of these terms can be

Table 2

Regression Statistics for the Calibration Curves of Theophylline

Parameter	(1) Peak Area	(2) Peak Height
Slope	38.2	1.896
C.I. slope	37.3 - 39.1	1.860 - 1.932
S.E. slope	0.4	0.016
Intercept	28	0.75
C.I. intercept	23, 33	0.71 - 0.79
S.E. intercept	2	0.10
Standard error	3.9	0.165
r	0.9982	0.9995
F	413	14086
N	16	16

* C.I. = Confidence intervals (95%);
S.E. = standard error;
r = correlation coefficient;
F = the ratio between the residual variance and the variance modelled by regression;
N = number of points

interpreted as high eluent strengths of the modifier and the surfactant, respectively. For theophylline the eluent strength of propanol was 16.063, 10.725 and 8.9454 for 0.05, 0.1 and 0.15 M SDS concentration, respectively, and the eluent strength of surfactant was + 0.5204, - 0.0134 and - 0.5473 for 0, 1.5 and 3% propanol concentration, respectively. These data should be interpreted in the following way. The eluent strength of propanol is significantly larger than the eluent strength of SDS.

Only for a purely aqueous SDS mobile phase (no propanol added) the SDS shows an appreciable eluent strength. If propanol is added to the mobile phase, the eluent strength of SDS is negligible.

Figure 1 shows the retention surface of theophylline as a function of the SDS and propanol concentrations. As can be observed, for a 0.05 M SDS mobile phase, an increase in propanol concentration from 0 to 3.0 % leads to a drastic decrease of retention. In the presence of a 3% propanol concentration, the increase of the SDS concentration in the mobile phase, practically, did not produced a decrease of the retention. Adequate retention and efficiency was achieved with a 0.05 M SDS + 3% propanol eluent and was selected for further experiments.

Table 3

Analysis of Pharmaceutical Preparations

Preparation (presentation), Source	Declared	Found (Peak Areas)	Found (Peak Heights)
Dexa-bronchisan (tablets), Boehringer Mannheim	Theophylline 50 mg Dexamethasone 0.5 mg. Diphenhidramine HCL 10 mg Ephedrine HCL 25 mg Calcium lactate 70 mg Excipient	36.3 ± 1.1	39. 6 ± 1.1
Eufilina retard 175 (covered tablets), Elmu	Theophylline 140.9 mg Ethylenediaminedichlorohydrate 75.5 mg Excipient	123 ± 2	125 ± 3
Elixifilin (syrup), Morrith S.A.	Theophylline 5.33 mg/mL Potassium iodure 8.66 mg/mL Saccharin sodium 0.0 mg/mL Sacharose 150 mg/mL Excipient and ethanol	5.23 ± 0.17	4.91 ± 0.08
Muco-teolixir (syrup), Carulla-Vekar S.A.	Theophylline 4 mg/mL N-acetyl-DL-homocysteine Thiolactone 0.04 mg/mL Sodium benzosulfimide 0.1 mg/mL Excipient	3.9 ± 0.1	3.86 ± 0.13
Teolixir compositum (syrup), Biogalenica S.A.	Theophylline 5.33 mg/mL Prednisolone 0.333 mg/mL Guaifenesin 6.66 mg/mL Saccharin sodium 1 mg/mL Ethanol 0.2 mL/mL Saccharose 225 mg/mL Excipient	5.20 ± 0.13	4.94 ± 0.06
Pulmeno (capsules), Sandoz Pharma S.A.	Theophylline anhydrous 200 mg Excipient	183 ± 4	179 ± 5

Analytical Data

The calibration curve of theophylline were obtained by triplicate injection of standard solutions with a varying concentration of the theophylline in the range 2-10 μg/mL. Peak heights and peaks areas were used as dependent variables.

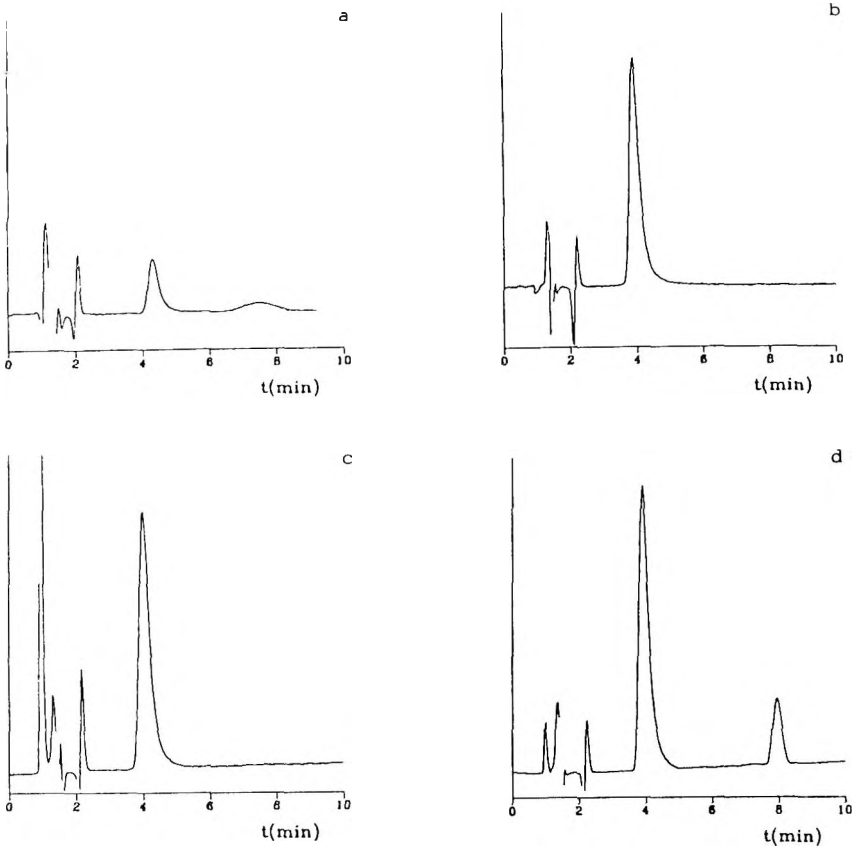

Figure 2. Chromatograms of some pharmaceutical preparations: a) Dexabronchisan; b) Eufilin retard; c) Mucoteolixir; d) Teolixir.

The presence of outliers, normality of residuals (Kolmogoroff test), homogeneity of variances (Cochran and Bartlett tests) and validity of the linear model (lack-of-fit test) were studied in agreement with the suggestions reported by Sarabia and Ortiz[16]

In all cases the significance levels found assures the validity of the regression models. Table 2 shows regression statistics for the calibration curves of theophylline.

The calibration curves showed adequate regression coefficients with peak areas and heights over the working interval. The value of the residual variance to the variance modelled by regression ratio (F) indicated that the use of peak height as dependent variable is preferable.

The reproducibility was evaluated from two series of five aliquots of theophylline. The coefficient of variation was 1.7% at a 3.8 μg/mL concentration level, and 1.2% for 7.6 μg/mL.

Analysis of Pharmaceutical Formulations

The procedure was applied to the determination of theophylline in six pharmaceutical preparations found in the spanish market, which contain theophylline together with a number of other components (Table 3). Some chromatograms are shown in Figure 2. As can be observed, the peaks of the others components in the samples did not overlap with the peak of theophylline.

The theophylline content was obtained by taking three aliquots of each three or five independent dissolved formulations, and injected into the chromatograph. The results were reproducible and the recoveries with respect to the values declared by the manufacturers were in the 85-104 % range using peak areas and between 83-97% using peak heights, except for Dexa-bronchisan (73% and 79%, respectively).

The proposed procedure for the determination of theophylline is rapid (five minutes per sample), reliable and free of interferences.

ACKNOWLEDGEMENTS

The authors are grateful to M. Cuenca, A. Montesinos and Y. Lopez for their collaboration in this work.

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Received November 24, 1995
Accepted December 20, 1995
Manuscript 4046

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1967–1991 (1996)

THE EFFECT OF CHROMATOGRAPHIC CONDITIONS ON THE RETENTION INDICES OF FORENSICALLY RELEVANT SUBSTANCES IN REVERSED-PHASE HPLC

Moutian Wu,* Rolf Aderjan

Institute of Forensic Medicine
University of Heidelberg
Vorßstraße 2
D-69115 Heidelberg, FRG

ABSTRACT

This paper deals with the effect of chromatographic conditions, such as the columns with different batches and lengths, buffer concentration in eluents, gradient profiles, pH-values of buffer and flowrates of the elution, on the retention indices of forensically relevant substances in reversed phase HPLC. Our study shows that retention index is only a method of linear correction. When the retention times of the analytes change, under deviation of chromatographic conditions, proportionally to that of the scale substances, the retention indices can well balance the variation from retention times.

* Present address: Institute of Forensic Sciences, Ministry of Justice, P.R.C., 1347 Guang Fu Xi Lu, Shanghai, 200063, P. R. China.

INTRODUCTION

High performance liquid chromatography (HPLC) can be widely used in quantitative analysis. However, its application to systematic toxicological analysis (STA) has been limited. This may partly arise from the poor reproducibility of the retentions of analytes, which makes it difficult to collect from HPLC retention data as useful to the identification of unknown substances in different situations as those collected from thin-layer chromatography (TLC)[1] and gas chromatography (GC).[2] This led us to investigate the factors that influence the reproducibility of retentions in HPLC.

The most reproducible results were obtained when the retentions were recorded as relative values,[3,4] either as relative capacity factors, corrected capacity factors, relative retention times, or as retention indices. As capacity factors have been conventionally calculated as $k' = (t_r - t_0)/t_0$, the values of k' are susceptible to the smallest changes of the column void volume (t_0). Many methods have been proposed to determine this value,[5-7] but they often give different results with the same column and equipment. Relative retention times compared with an internal standard is a simpler method, but each laboratory may have different standard compounds, so that direct comparison of the results are impossible.

Kovats' retention indices have been widely used in GC because they are more comparable than direct retention times under different chromatographic conditions, but similar concepts have not been accepted in HPLC, so far. Since the first proposals made by Baker and Ma,[8] who suggested that the alkan-2-ones could be used as a scale for retention indices in HPLC, Smith[9] has suggested that alkyl aryl ketones would be more easily detected with UV as a retention indices scale. R. Aderjan and M. Bogusz,[10,11] have put forward 1-nitroalkane as retention indices scale both for GC and HPLC. A series of studies aimed at improving the reproducibility of retention values in HPLC have been made.[12] The influence of the eluent composition,[13] instruments setup,[14] operating temperature and the nature of the stationary phase[15] on the retention of barbiturates, local anaesthetic drugs, basic drugs and thiazide diuretics[16] with related drugs in reversed-phase HPLC has been studied. The results showed that the retention indices of neutral sample compounds were virtually independent of proportion of methanol-water in eluents over a wide range, and the retention indices of basic drugs and the references not affected in the same way by the chromatographic conditions because basic drugs have so many different chemical structures. M. Bogusz et al. used a method of correction to improve the reproducibility of retention indices in gradient elution between RP-18 columns using different groups of standards for neutral/acidic drugs or basic drugs.[17,18] M. Bogusz and M. Wu[19] used the retention indices based on 1-nitroalkane to standardize HPLC system for STA. Recently, 1-nitroalkane has been also applied[20] to the retention indices for STA in the reversed-phase HPLC.

However, most of these studies on retention indices in HPLC were done using isocratic elution and investigating neutral/acidic or basic substances with different eluents or corrected with different standards. No corresponding study has been made of the effect of changing the chromatographic conditions on the retention indices with the elution system, which is suitable for STA.

The present study is a systematic examination of the applicability of the retention indices, based on 1-nitroalkane to acetonitrile-phosphate buffer gradient elution. One aim of the study is to determine the robustness of the retention indices to small changes in chromatographic conditions and to identify the factors that must be strictly controlled in order to obtain consistent results from different laboratories.

Our work includes a detailed examination of more than 100 substances of forensic interest with various chemical structure classes in gradient elution.

EXPERIMENTAL

Instruments

Experiments were carried out with an H/P HPLC system (Hewlett-Packard, Avondale, PA, USA) equipped with a Model 1050 series pump and autosampler, HP 300 Chemstation and HP 1040 DAD detector. The DAD detector was set up at 220 nm as monitor wavelength. A LiChroCART column (125mm x 4mm ID) packed with 4 µm Supspher 100 RP-18 (Merck, FRG) was used. A guard column (4 x 4 mm), filled with the same material was installed.

The saturation column, filled with Lichrospher RP-18 was mounted between the pump and the injector to provide protection of the analytical column against the influence of amine modifier.

Chemicals

Drugs involved in this study were diluted with methanol to a concentration of 50-100 µg/mL. A series of 1-nitroalkanes—nitromethane, nitroethane, 1-nitrobutane, 1-nitropentane and 1-nitrohexane—was obtained from Fluka AG, Switzerland. 1-Nitroheptane and 1-nitrooctane were synthesized as previously[21]. Acetonitrile was analytical grade and obtained from Roth Gmbh, FRG. Triethylammoniumphosphate buffer (1 M in water) was supplied by Fluka.

Table 1

Reproducibility of Retention Times and Peak Shape of 1-Nitroalkane during Four Months

Homolog*	Rt±SD (min)	CV%	Width±SD	CV%	Symmetry±SD	CV%
C1	2.094±0.015	0.716	0.147±0.10	6.8	0.511±0.041	8.0
C2	5.168±0.047	0.909	0.150±0.014	9.3	0.533±0.033	6.2
C3	10.760±0.092	0.855	0.210±0.022	10.5	0.789±0.147	18.6
C4	17.775±0.135	0.759	0.184±0.019	10.3	0.857±0.099	11.6
C5	22.181±0.137	0.618	0.159±0.014	8.8	0.847±0.089	10.5
C6	25.479±0.134	0.526	0.150±0.011	7.3	0.883±0.084	9.5
C7	28.181±0.127	0.451	0.148±0.010	6.8	0.807±0.078	9.7
C8	30.638±0.125	0.408	0.151±0.011	7.2	0.613±0.083	10.2

* C-Atomic number of 1-nitroalkane (n = 20).

HPLC Conditions

The HPLC buffer was prepared by adding 25 mL triethylammonium phosphate buffer to 1000 mL water. The pH was about 3.1. The elution was followed by the acetonitrile buffer linear gradient: at the beginning 0% acetonitrile, after 30 min 70% acetonitrile, keeping 70% acetonitrile for 5 min, 10 min of the post time. The flow rate was 1 mL/min and the injection volume 10 μL. The above conditions were used as our standard system in this paper.

RESULTS AND DISCUSSION

Reproducibility of the System

Using the standard system, without any changes in chromatographic conditions, we have observed the retention behaviour of 1-nitroalkane and a set of test solutions for four months. The test solutions included neutral, acidic and basic substances, respectively, which, when chromatographed over a wide range, covered nearly all the important areas of the gradient elution. The results are shown in Table 1 and Table 2.

Not only the retention times but also the other chromatographic properties of 1-nitroalkane, such as the width and the symmetry factor of the peak, were reproducible. All the CV% values for retention times of 1-nitroalkane were

Table 2

Reproducibility of Retention Times and Retention Indices of Acidic, Neutral and Basic Substances in Mixed Solutions (n=20)

Substance	Rt±SD(min)	CV%	RI±SD	CV%
Paracetamol	7.05±0.14	1.99	234±1.9	0.812
Barbital	10.05±0.14	1.39	287±1.8	0.627
Brallobarbital	14.79±0.16	1.08	359±1.6	0.446
Pentobarbital	17.89±0.16	0.894	405±1.9	0.469
Secobarbital	19.15±0.15	0.783	437±1.6	0.366
Clobazam	21.31±0.20	0.939	484±2.2	0.455
Indometacine	25.64±0.25	0.975	610±3.3	0.541
Prazepam	26.79±0.12	0.448	648±3.5	0.540
Morphine	5.12±0.09	1.76	198±1.9	0.960
Chloroquine	8.80±0.07	0.795	265±0.5	0.189
Benzoylecgonine	10.52±0.06	0.570	295±0.8	0.271
Cocain	13.34±0.12	0.900	336±1.9	0.565
Diphenydramine	16.75±0.13	0.776	385±2.0	0.519
Haloperidol	18.11±0.15	0.828	409±1.7	0.416
Amitriptyline	19.69±0.03	0.152	446±2.7	0.605
Thioridazine	22.32±0.13	0.582	504±3.9	0.774
Meclozine	25.41±0.22	0.866	601±3.0	0.499
Amiodaron	29.65±0.36	1.21	762±4.4	0.577

smaller than 1.0%, 0.66% in average. The width and symmetry factor have a same level of CV% values. These width and symmetry factors demonstrate that the theoretical plate number of the column has not greatly changed after the long term run.

For the substances in test solutions, shown in Table 2, the average of CV% of retention times was 1.2%, which is greater than the average of that of retention indices, 0.66%. The reproducibility of the retention times of 1-nitroalkane was better than that of the substances in test solutions.

During the four months, we kept the instruments simply at ambient temperature (22 °C±4 °C). No serious influence of operating temperature has been found. R. M. Smith has reported that, over a small range (±5 °C), the influence is small (<10 RI units),[16] and is not likely to interfere with identification procedures. In his paper we can see that, for cyclobarbitone, for

Table 3

Characteristics of the Columns

No.	Length x i.d.	Neff./m*	Rt (C8, min)±SD**	CV%	Flowrate (mL/min)
1	125 x 4	3731	30.600±0.02	0.07	1.0
2	125 x 4	1892	30.049±0.04	0.13	1.0
3	125 x 4	4456	30.411±0.06	0.20	1.0
4	125 x 4	2441	29.920±0.12	0.40	1.0
5	50 x 4	1273	25.863±0.03	0.12	0.6

Mark: Lichrocart; Manufacturer: Merck AG; Packing material: Superspher 100 RP 18, 5 μm for columns No. 1-4, 4 μm for column No. 5
* Calculated by 1-Nitroheptane with isocratic elution, 60:40 acetonitrile:TEAP-buffer
**Calculated with n=20 for columns No. 1-4, n=16 for column No. 5
Neff./m: theoretical plate

Table 4

Linear Relationship Coefficients between Retention Time or Retention Indices on Different Lengths of Columns

Item	A	B	R
Retention time of 1-nitroalkane	0.906	-2.80	0.984
Retention indices of substances in the test solutions	0.942	62.84	0.991
Retention time of substances in the test solutions	0.902	-1.86	0.993

Linear Relationship: $Y = A*X + B$
Y: Data on the 5 cm column; X: Data on the 12.5 cm column.
R: Correlation Coefficient

instance, ΔRI was 12, 6, 10, 15 between 10 °C and 20 °C, 20 °C and 25 °C, 25 °C and 30 °C, 30 °C and 40 °C, respectively. In our work, we found temperature played an even smaller role on the retention. The reproducibility of retention times of 1-nitroalkane and the above listed analytes and of the width and symmetry factor are proof of high stability of the system, of both the equipment and the elution conditions, which is very important for gradient elution and our later investigation. In spite of this, for greater guarantee of

control we added 1-nitrooctane to each sample to monitor the reproducibility of each gradient run. And this confirmed that we could use the system to investigate the influence of small changes in operating conditions on the retention of the 1-nitroalkane scale and solutes.

Columns with Different Batches and Lengths

We know that, in interlaboratory comparisons, an important cause of irreproducibility is the differences between nominally equivalent C_{18} bonded silicas. It was reported that differences include minor, but significant, ones between batches from the same manufacturer and much bigger ones between manufacturers.

Four columns, filled with the same packing material from one manufacturer were used in our study. Their characteristics are given in Table 3. Column 3 was new and column 4 very old. Both columns 1 and 2 have been long used for our routine analysis for over a year. The batch number of the commercial columns was different in each case.

All the substances examined with the first four columns were well reprodu-cible, both in retention indices and in retention times. The greatest CV% of retention indices was 2.55% with SD = 8.18 for phenazone. This deviation is acceptable for routine analysis. It is more surprising that, with this HPLC system, no significant differences in retention times were found with different batches of the same brand, although these columns have different values of Neff/m.

The linear coefficients of the relationships between retention times or retention indices on columns 5.0 cm and 12.5 cm, shown in Fig.1, is given in Table 4. We can see from the results that there is no difference between these linear relationships when the retentions on the 5.0 cm and 12.5 cm column were expressed with retention indices and with retention times.

When the 5 cm column, which was identical with the 12.5 cm columns except for the length of the columns, was put into use with the same gradient but different flow rate (0.6 mL/min), linear consistencies were observed (Fig.1). The linear coefficient was r = 0.991 for retention indices and r=0.993 for retention times of the over 100 substances between 5cm and 12.5cm columns.

The relationship regressed with the least squares method as listed in Table 4. From Table 4, we could find that the linear relationship of the examined substances was better than that of 1-nitroalkane. For the earlier eluted substances, the reproducibility of the retention times from the 5 cm and 12.5 cm

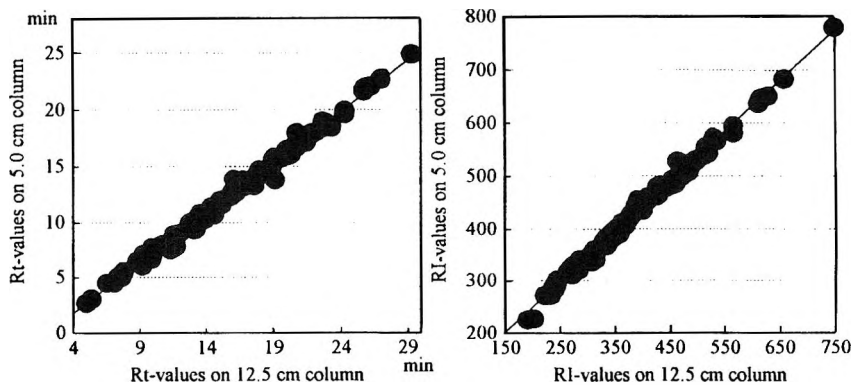


Figure 1. Linear relationships between retention times, retention indices, on 5 cm column and on 12.5 cm column.

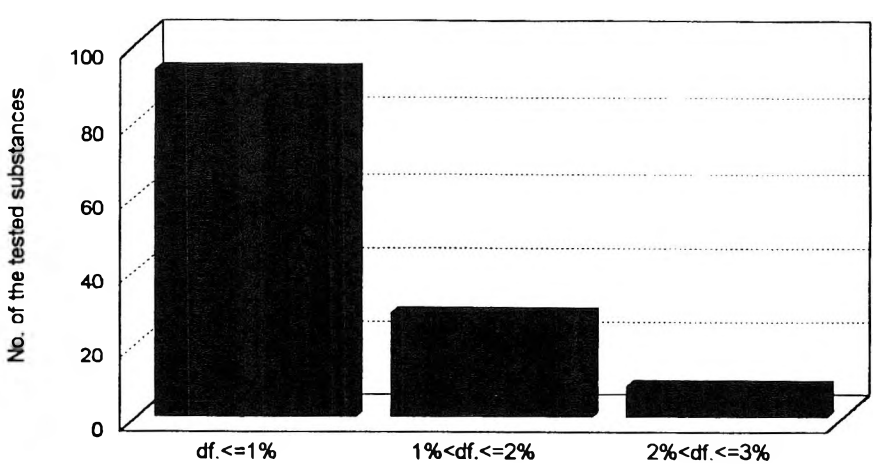


Figure 2. Distribution of the differences (df.) in retention times of the tested substances eluted with different TEAP-concentrations

Table 5

Retention Times of 1-Nitroalkane Eluted with Different TEAP-Concentrations in Eluents

Homolog*	Retention time (min)			Ev**-Rt±SD	CV%
	25 mL	20 mL	30 mL		
C 1	2.176	2.185	2.243	2.201±0.036	1.65
C 2	5.233	5.398	5.454	5.362±0.115	2.14
C 3	10.807	10.779	10.970	10.852±0.103	0.95
C 4	17.549	17.389	17.616	17.518±0.117	0.67
C 5	22.149	22.033	22.067	22.083±0.060	0.27
C 6	25.425	25.361	25.332	25.373±0.048	0.19
C 7	28.133	28.069	28.031	28.078±0.052	0.18
C 8	30.595	30.493	30.467	30.519±0.068	0.22
Ev C 8 (n=20)	30.599	30.329	30.464		
SD C 8 (n=20)	±0.015	±0.111	±0.082		
CV%	0.049	0.37	0.27		

* C-Atomic number of 1-nitroalkane.

**Average of rentention time.

columns was somewhat poorer, because nitroethane and 1-nitropropane were chromatographed later than they would be in an ideal linear relationship.

TEAP-Buffer Concentration in Eluents

It is well known that drugs with structures containing basic nitrogen atoms can show tailing peaks in reversed-phase HPLC. These problems are recognised to arise from interactions between the drugs and the adsorption sites on the silica matrix of the packing material.[22,23]

An eluent with modifier is necessary for STA in order to get sharper and more symmetrical peaks for basic substances. Triethylammonium phosphate (TEAP) has high solubility in aqueous eluents and can be used as part of the buffer system..

The effect of different TEAP concentrations in eluents on the retention times and retention indices was investigated. We changed the TEAP-concentration from 20 mL to 30 mL of 1 M TEAP in 1 L eluent, but kept all other HPLC conditions constant, then the 1-nitroalkane and over 100 forensic relevant substances were chromatographed.

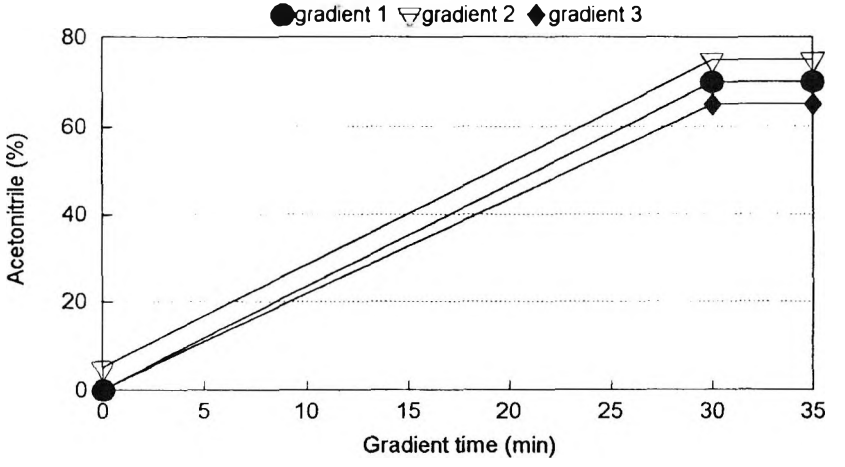


Figure 3. Three different gradient profiles used.

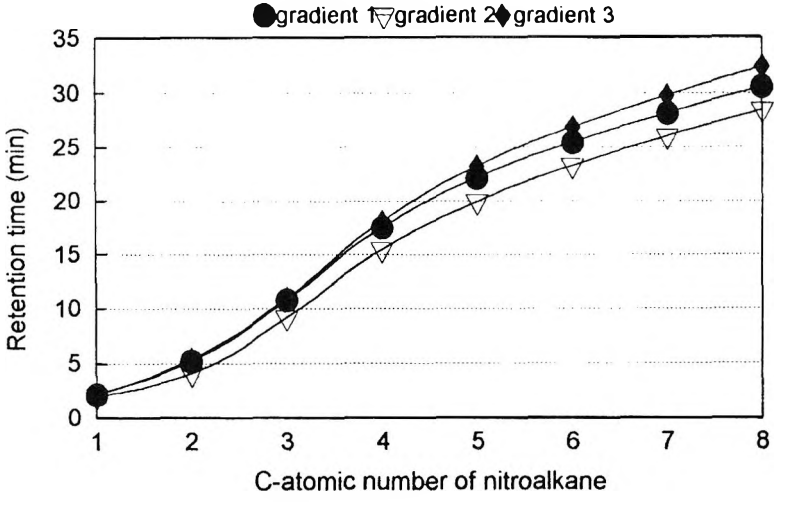


Figure 4. The retention times of 1-nitroalkane under the three different gradient profiles.

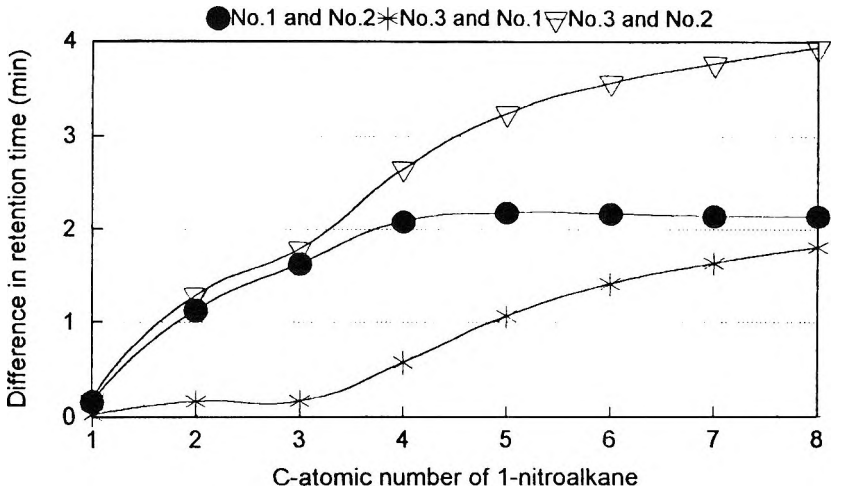


Figure 5. Differences in the retention times of 1-nitroalkane under gradient No. 1 and No. 2, that under No. 3 and No. 1 and that under No. 3 and No. 2.

The retention times of 1-nitroalkane with different concentrations of TEAP-buffer was shown in Table 5. Figure 2 showed that almost all retention times of the tested substances, eluted with different TEAP concentrations, did not change greatly. The difference of the retention times of the tested substances, eluted with three different TEAP concentrations, were mostly (72%) smaller than 1%, 22% of them between 1% and 2%, 6% of them between 2% and 3%, none of them greater than 3%. The TEAP concentrations played no significant role on the retention behaviours of 1-nitroalkane and of the tested substances.

Effect of Gradient Profiles

One of the most important causes which strongly affects the retention times in gradient elution HPLC, is the reproducibility of gradient profile. A slight change in the components of eluent on line may produce great deviations in retention times. Sometimes, in different circumstances, the HPLC systems were run under nominally identical but, in fact, under slightly varied conditions, such as, the gradient profile, the flowrate, etc.

P. Jundere and J. Churacek[24] thought that the intercept, the slope and the concentration at the beginning would be the most important factors of a linear gradient profile, which could affect the retention times in the HPLC. We used

three slightly different linear gradient profiles in order to investigate how the gradient profiles would affect retention times and retention indices. The three different gradient profiles were given in Figure 3.

The retention times of 1-nitroalkane under three different gradient profiles were given in Figure 4. As expected, there was no significant variation in retention times of nitromethane, nitroethane, 1-nitropropane and nitrobutane under gradient No. 1 and No. 3 because of their similarity during the first 15 min of gradient profiles.

Under these conditions, we would have almost the same retention times of the tested substances and, consequently, the same retention indices.

But later, the difference in retention times grew bigger with the difference in both the gradient profiles. There was a relationship between the difference in the retention times obtained under gradient No. 1 and No. 3 and the C-atomic number of 1-nitroalkane.

Over 1-nitrobutane (retention times about 17 min) the difference in retention times was constant. (See Figure 5.)

This implies that retention indices can well balance the variations in retention times which were caused by the unidentical repeat of gradient profile under different circumstances. As can be seen from the results in Table 6, a comparison of the difference in retention times and retention indices under three different gradient profiles showed that the reproducibility of retention indices was much better than that of retention times.

The average of difference in retention times of the 113 substances tested was 10.9%, but that of retention indices only 2.07%. All the substances with relatively larger variation in the retention times under gradient No.1 and No.2, for example morphine, procainamid etc., were eluted sooner under gradient No.2 than under No.1, while under the same conditions nitromethane and nitroethane were not.

In this situation, retention indices of morphine and procainamid, etc., could not balance the difference in the retention times so well as those of the other substances, such as atenolol, paracetamol, etc., which were eluted somewhat later and whose retention times changed in proportion to the changes in retention times of 1-nitroalkane.

Some substances, such as trifluoperazine and parathion, etc., eluted under gradient No.1 and No.3, had relatively larger differences in

Table 6

Retention Times and Retention Indices of 113 Substances under the Three Different Gradient Profiles, No. 1, No. 2, and No. 3

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Acebutolol	RT:	11.58	9.41	12.08	11.02±1.42	12.9
	RI:	311	304	316	310±6.0	1.94
Acetanilide	RT:	11.68	9.67	12.25	11.20±1.35	12.1
	RI:	313	308	318	313±5.0	1.60
Alimenazin	RT:	13.41	11.40	14.27	13.03±1.47	11.3
	RI:	338	335	346	340±5.7	1.67
Allobarbital	RT:	13.14	10.84	13.55	12.51±1.46	11.7
	RI:	334	326	336	332±5.3	1.59
Alprazolam	RT:	19.97	18.09	21.09	19.72±1.52	7.69
	RI:	452	458	458	456±3.5	0.76
Alprenolol	RT:	16.09	14.14	17.08	15.77±1.50	9.49
	RI:	378	379	385	381±3.8	0.99
Aminophenazon	RT:	7.57	5.44	7.74	6.92±1.28	18.5
	RI:	241	226	242	236±9.0	3.79
Amitritylin	RT:	20.21	18.46	21.47	20.05±1.51	7.50
	RI:	458	466	464	463±4.2	0.90
Amobarbital	RT:	17.94	15.76	18.93	17.54±1.62	9.24
	RI:	409	406	416	410±5.1	1.25
Aprobarbital	RT:	13.93	11.88	14.62	13.48±1.43	10.6
	RI:	346	342	350	346±4.0	1.16
Aspirin	RT:	13.06	10.91	13.85	12.61±1.52	12.1
	RI:	333	327	340	333±6.5	1.95
Atenolol	RT:	6.49	4.16	6.79	5.89±1.44	24.8
	RI:	223	201	225	216±13	6.16
Azinphos-Methyl	RT:	24.28	22.26	25.50	24.01±1.64	6.81
	RI:	565	569	563	566±3.1	0.54
Barbital	RT:	9.92	7.20	10.39	9.17±1.72	18.8
	RI:	284	261	289	278±15	5.38
Benzoylecgonine	RT:	10.60	8.48	11.16	10.08±1.41	14.0
	RI:	296	286	303	295±8.5	2.90
Brallobarbital	RT:	14.64	12.74	15.27	14.22±1.32	9.26
	RI:	357	357	360	358±1.7	0.48
Bromazepam	RT:	16.22	14.12	17.11	15.82±1.54	9.72
	RI:	380	378	385	381±3.6	0.95

(continued)

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Butabital	RT:	16.09	14.06	16.91	15.59±1.35	8.64
	RI:	378	378	383	380±2.9	0.76
Camazepam	RT:	23.95	21.92	24.81	23.56±1.48	6.30
	RI:	554	559	544	552±7.6	1.38
Carbamazepin	RT:	16.91	15.94	19.03	17.29±1.58	9.14
	RI:	391	410	418	406±14	3.42
Chlordiazepoxide	RT:	14.71	12.70	15.48	14.29±1.44	10.0
	RI:	358	356	363	359±3.6	1.00
Chloroquin	RT:	8.83	6.61	9.16	8.20±1.39	16.9
	RI:	265	249	267	260±9.9	3.69
Chlorprothixen	RT:	21.74	20.01	23.08	21.61±1.54	7.12
	RI:	491	501	498	497±5.1	1.03
Clobazam	RT:	21.77	19.26	23.23	21.42±2.01	9.37
	RI:	492	484	503	493±9.5	1.93
Clomipramin	RT:	21.38	17.86	22.51	20.58±2.43	11.8
	RI:	483	497	488	489±7.1	1.45
Clonazepat	RT:	19.61	17.54	20.66	19.27±1.59	8.24
	RI:	445	446	450	447±2.6	0.59
Clopamid	RT:	14.76	12.57	15.41	14.25±1.49	10.4
	RI:	357	354	361	357±3.5	0.98
Cocain	RT:	13.45	11.56	14.35	13.12±1.42	10.9
	RI:	339	338	347	341±4.9	1.45
Caffein	RT:	9.37	7.20	9.92	8.83±1.44	16.3
	RI:	274	261	281	272±10	3.73
Codein	RT:	7.7	5.61	8.09	7.14±1.34	18.7
	RI:	244	230	248	241±9.5	3.93
Cyclopentabarbitol	RT:	15.91	13.90	16.78	15.53±1.48	9.5
	RI:	376	375	381	377±3.2	0.85
Diazepam	RT:	23.27	21.21	24.33	22.94±1.59	6.92
	RI:	534	538	531	534±3.5	6.57
Diazoxid	RT:	13.88	11.62	14.55	13.35±1.54	11.5
	RI:	346	339	350	345±5.6	1.61
Dibenzepin	RT:	14.38	12.46	15.29	14.04±1.44	10.3
	RI:	353	352	360	355±4.4	1.23
Diclofenac	RT:	25.72	23.72	27.15	25.53±1.72	6.75
	RI:	611	616	611	613±2.9	0.47
Dimethoat	RT:	13.19	10.99	13.90	12.69±1.52	12.0
	RI:	335	329	341	335±6.0	1.79

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Diphenhydramin	RT:	17.19	15.19	18.25	16.88±1.55	9.21
	RI:	395	396	402	398±3.8	0.95
Dipyridamol	RT:	16.66	14.61	17.48	16.25±1.48	9.10
	RI:	387	386	391	388±2.6	0.68
Doxepin	RT:	17.82	15.95	18.95	17.57±1.52	8.62
	RI:	406	410	416	411±5.0	1.23
Dosulepin	RT:	19.07	17.13	20.36	18.85±1.63	8.62
	RI:	433	436	444	438±5.7	1.30
Ethenzamid	RT:	14.56	12.59	15.41	14.19±1.45	10.2
	RI:	356	354	362	357±4.2	1.17
Fenbufen	RT:	22.67	20.55	24.00	22.41±1.74	7.77
	RI:	516	518	521	518±2.5	0.49
Flecainid	RT:	18.03	16.12	19.06	17.74±1.49	8.41
	RI:	410	414	418	414±4.0	0.97
Flunitrazepam	RT:	20.72	18.72	21.74	20.39±1.54	7.53
	RI:	468	472	471	470±2.1	0.44
Fluphenazin	RT:	23.14	21.18	24.19	22.84±1.53	6.69
	RI:	530	537	527	531±5.1	0.97
Flurazepam	RT:	16.96	14.95	18.00	16.63±1.55	9.31
	RI:	391	392	398	394±3.8	0.96
Furosemid	RT:	17.96	16.07	19.09	17.71±1.53	8.62
	RI:	409	413	419	414±1.22	1.22
Glibenclamid	RT:	25.81	23.84	27.14	25.60±1.66	6.49
	RI:	615	621	611	616±5.0	0.82
Glipizid	RT:	20.39	18.22	21.57	20.06±1.70	8.47
	RI:	462	461	469	464±4.4	0.94
Gliquidon	RT:	28.81	26.64	30.43	28.63±1.40	6.64
	RI:	726	726	731	728±2.9	0.40
Heptabarbital	RT:	17.62	20.01	18.50	18.71±1.21	6.46
	RI:	401	402	407	403±3.2	0.80
Hydrochlorothiazid	RT:	9.18	7.10	9.80	8.69±1.41	16.3
	RI:	271	259	279	270±10.1	3.73
Ibuprofen	RT:	26.20	24.07	27.71	26.00±1.83	7.05
	RI:	628	629	630	629±1.0	0.16
Idobutal	RT:	17.08	14.87	17.95	16.63V1.59	9.55
	RI:	393	391	398	394±3.6	0.92

(continued)

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Imipramin	RT:	19.46	17.56	20.82	19.28±1.64	8.49
	RI:	442	446	453	447±5.6	1.25
Ketotifen	RT:	15.10	13.00	16.03	14.71±1.55	10.6
	RI:	363	361	370	365±4.7	1.30
Linuron	RT:	24.26	22.23	25.49	23.99±1.65	6.86
	RI:	565	570	563	566±3.6	0.64
Lorazepam	RT:	19.11	17.06	20.22	18.80±1.60	8.53
	RI:	434	435	441	437±3.8	0.87
Lormethazepam	RT:	21.46	19.29	22.66	21.14±1.71	8.08
	RI:	485	485	491	487±3.5	0.71
Meclozine	RT:	26.66	24.94	28.31	26.64±1.69	6.33
	RI:	645	662	650	652±8.7	1.34
Medazepam	RT:	17.57	15.73	18.76	13.35±1.53	8.80
	RI:	400	405	412	406±6.0	1.49
Mescalin	RT:	19.14	17.21	2-.31	18.89±1.57	8.29
	RI:	435	439	443	439±4.0	0.91
Metamizol	RT:	9.95	7.71	10.37	9.34±1.43	15.3
	RI:	285	271	289	282±9.5	3.36
Metoclopramid	RT:	11.38	9.47	12.15	11.00±1.38	12.5
	RI:	309	305	316	310±5.6	1.80
Metronidazol	RT:	7.30	5.14	7.66	6.70±1.36	20.3
	RI:	237	220	241	233±11.2	4.79
Mianserin	RT:	17.28	15.35	18.50	17.04±1.59	9.32
	RI:	396	398	405	400±4.7	1.18
Midazolam	RT:	16.85	14.88	17.94	16.56±1.55	9.37
	RI:	389	390	398	392±4.9	1.26
Morphin	RT:	4.95	2.68	5.04	4.22±1.34	31.7
	RI:	191	132	189	171±33.5	19.6
Nadolol	RT:	9.28	7.12	9.73	8.71±1.40	16.0
	RI:	273	259	278	270±9.8	3.65
Nafopam	RT:	14.82	12.82	15.83	14.49±1.53	10.6
	RI:	360	358	368	362±5.3	1.46
Nalorphin	RT:	7.49	5.32	7.89	6.90±1.38	20.0
	RI:	240	224	244	236±10.6	4.48
Naproxen	RT:	20.72	19.81	22.84	21.12±1.55	7.36
	RI:	493	495	493	494±1.2	0.23

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Nifedipin	RT:	22.13	20.09	23.43	21.88±1.68	7.69
	RI:	500	503	506	503±3.0	0.60
Nitrazepam	RT:	18.86	16.75	19.94	18.52±1.62	8.76
	RT:	427	428	436	430±4.9	1.15
Nordiazepam	RT:	20.51	18.49	21.73	20.24±1.64	8.08
	RI:	464	467	471	464±3.5	0.75
Noscapin	RT:	14.70	12.68	15.63	14.34±1.51	10.5
	RI:	358	356	365	360±4.7	1.31
Opipramol	RT:	16.52	14.50	17.43	16.16±1.50	9.27
	RI:	3385	385	390	387±2.9	0.75
Orphenadrin	RT:	18.83	16.89	20.00	18.57±1.57	8.45
	RI:	427	431	437	432±5.0	1.17
Oxazepam	RT:	18.64	16.54	19.57	18.25±1.55	8.51
	RI:	423	424	428	425±2.6	0.62
Oxyphenbutazon	RT:	21.92	19.77	23.27	21.65±1.77	8.15
	RI	495	496	502	498±3.8	0.76
Papaverin	RT:	14.27	12.32	15.19	13.93±1.47	10.5
	RI:	351	350	359	353±4.9	1.40
Paracetamol	RT:	7.06	4.76	7.17	6.33±1.36	21.5
	RI:	233	213	232	226±11.3	4.99
Paraoxon	RT:	21.39	19.42	22.51	21.11±1.56	7.41
	RI:	483	488	486	486±2.5	0.52
Parathion	RT:	29.29	27.32	30.06	28.56±1.08	3.77
	RI:	747	754	711	759±15.7	2.07
Pemolin	RT:	9.88	7.65	10.12	9.22±1.36	14.8
	RI:	283	270	284	279±7.8	2.80
Pentazocin	RT:	15.03	12.94	15.86	14.61±1.50	10.3
	RI:	363	360	369	364±4.0	1.11
Pentobarbital	RT:	17.77	15.92	18.65	17.45±1.39	7.99
	RI:	405	405	411	407±3.5	0.85
Perphenazin	RT:	20.00	18.09	21.21	19.77±1.57	7.96
	RI:	453	458	460	457±3.6	0.79
Phenacetin	RT:	14.78	12.75	15.55	14.36±1.45	10.1
	RI:	358	357	364	360±3.8	1.05
Phenazon	RT:	11.69	9.64	12.33	11.22±1.41	12.5
	RI:	313	307	319	313±6.0	1.92

(continued)

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Phenylbutazon	RT:	26.96	24.86	28.67	26.83±1.91	7.11
	RI:	657	658	662	659±2.6	0.49
Pindolol	RT:	9.53	7.49	10.06	9.03±1.36	15.0
	RI:	277	267	283	276±9.1	2.93
Prazepam	RT:	27.09	25.04	28.68	26.94±1.82	6.77
	RI:	661	665	668	665±3.5	0.53
Procain	RT:	7.39	5.22	7.71	6.77±1.35	20.0
	RI:	239	221	241	234±11.0	4.71
Procainamid	RT:	5.34	2.92	5.41	4.56±1.42	31.1
	RI:	202	143	200	182±33.5	18.4
Propranolol	RT:	15.90	13.93	16.83	15.55±1.48	9.52
	RI:	376	376	381	378±2.9	0.76
Protriptylin	RT:	19.07	17.15	20.25	18.82±1.56	8.31
	RI:	432	437	441	437±4.5	1.03
Quinidin	RT:	11.70	8.74	12.26	10.90±1.89	17.4
	RI:	313	291	318	307±14.4	4.67
Quinin	RT:	11.12	9.08	11.66	10.62±1.36	12.8
	RI:	306	298	309	304±5.7	1.87
Reserpin	RT:	31.43	19.50	23.00	21.31±1.75	8.23
	RI:	484	489	496	490±6.0	1.23
Salicylamid	RT:	11.31	9.05	11.58	10.65±1.39	13.0
	RI:	307	297	309	304±6.5	2.11
Secbutabarbital	RT:	15.14	12.94	15.93	14.67±1.55	10.6
	RI:	367	360	369	365±4.7	1.29
Sulpirid	RT:	7.29	4.99	7.54	6.61±1.41	21.3
	RI:	237	217	238	231±11.8	5.14
Temazepam	RT:	20.68	18.61	21.82	20.37±1.63	7.99
	RI:	468	470	473	470±2.5	0.54
Theophyllin	RT:	7.77	5.61	8.19	7.19±1.38	19.3
	RI:	245	230	250	242±10.4	4.30
Thioridazin	RT:	23.27	21.56	24.67	23.17±1.56	6.72
	RI:	543	548	540	541±7.0	1.30
Tolbutamid	RT:	20.73	18.66	21.89	20.43±1.64	8.01
	RI:	469	471	474	471±2.5	0.53
Triazolam	RT:	20.27	18.38	21.39	20.01±1.52	7.60
	RI:	459	465	464	463±3.2	0.69

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Trichlormethiazin	RT:	15.80	13.97	16.99	15.59±1.52	9.76
	RI:	374	376	384	378±5.3	1.40
Trifluoperazin	RT:	22.77	21.02	24.30	22.78±1.52	6.67
	RI:	519	532	530	527±7.0	1.33
Triflupromazin	RT:	22.62	20.46	23.93	22.34±1.75	7.84
	RI:	514	515	520	516±3.2	0.62
Viloxazin	RT:	11.97	10.01	12.70	11.56±1.39	12.0
	RI:	317	313	324	318±5.6	1.75
Vinylbital	RT:	17.87	15.75	18.80	17.47±1.56	8.95
	RI:	406	406	413	408±4.0	0.99

[a] RT = Retention Times.
RI = Retention Indices.

retention indices, while others, such as ibuprofen, prazepam, gliquidon and fenbufen, etc., did not.

The retention times of gliquidon changed from 30.43 min under gradient No.3 to 28.81min under gradient No.1 and that of the corresponding 1-nitrohepthane from 29.76 min to 28.13 min, so the retention indices of gliquidon stayed almost the same, with values of 731 and 726.

On the other hand, under the same situation, the retention times of parathion varied from 30.06 min to 29.29 min, so the retention indices of parathion, under gradient No.3 and No.1, which were 711 and 747 respectively, could not balance the difference in their retention times.

Effect of the pH Values in Eluent

The effect of the pH-values in the TEAP-buffer on the retention times and retention indices was tested by changing the pH value in the eluent and keeping all other conditions mentioned in the experimental section constant. The pH values were changed by adding 1 M NaOH to the eluent, up to pH 4 or pH 5.

During gradient elution, the pH values should be increased with the increase of percentage of acetonitrile in the eluent on line. The changes of pH values during gradient elution are linear as shown in Figure 6.

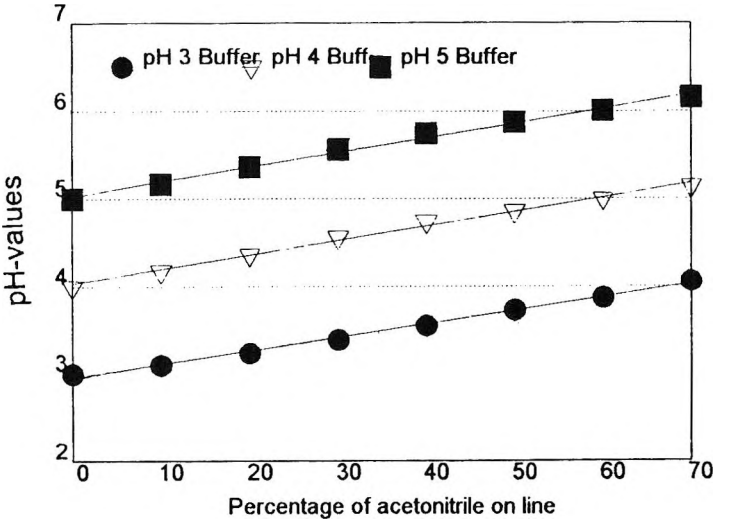


Figure 6. pH values of eluent during the gradient elution.

Table 7

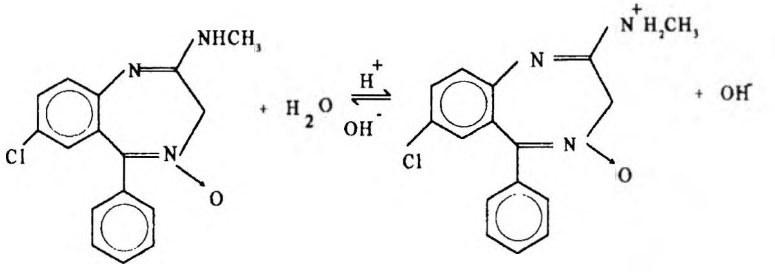
The Retention Times of 1-Nitroalkane under Three Different Elution Flow Rates

Homolog	Flowrate mL/min			Average	
	1.0	1.1	0.9	Rt (min)	CV%
C 1	2.176	2.005	2.459	2.214±0.239	10.4
C 2	5.233	4.936	5.844	5.338±0.463	8.71
C 3	10.807	10.175	11.657	10.880±0.744	6.83
C 4	17.549	16.926	18.306	17.594±0.691	3.93
C 5	22.149	21.684	22.873	22.235±0.599	2.69
C 6	25.425	25.028	26.167	25.540±0.578	2.26
C 7	28.133	27.729	28.883	28.248±0.586	2.07
C 8	30.595	30.151	31.344	30.685±0.585	1.91
MW C 8 (n=20)	30.599	30.135	31.310		
SD C 8 (n=20)	0.015	0.057	0.044		
CV%	0.049	0.189	0.141		

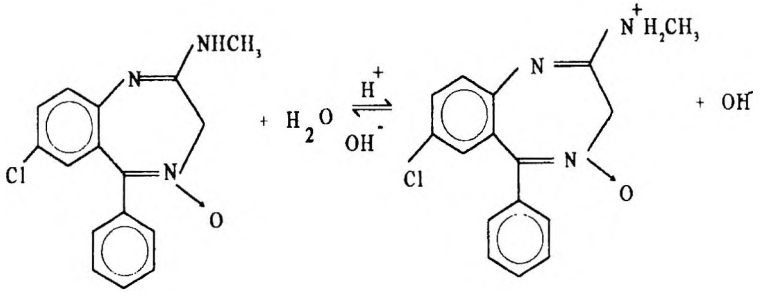
The retention times of the substances whose pK_a or pK_b values were in the range of the changes in pH values during the gradient elution, such as aspirin, chlordiazepoxide, quinine, etc., changed greatly when eluents with different pH values as in our test with pH 3, pH 4 or pH 5 were used. Under such conditions,

the retention indices of these substances could not stay the same because the retention times of the retention index scale used, 1-nitroalkane, changed only slightly.

The changes of the retention times of the substances mentioned above are related to their acid-base equilibria. For example, aspirin, with a pK_a value of 3.25, has the following acid-base equilibrium:



With the increase in the pH-values of the buffer used, the equilibria should move to the right in ion form. This may result in the decrease of the retention times of aspirin, because the ion form of aspirin is eluted more quickly than aspirin itself. On the other hand chlordiazepoxide has a different acid-base equilibrium:



and a pK_b value of 4.6. The ion form of chlordiazepoxide may be chromatographed more quickly too. With an increase in the pH of the buffer, the equilibrium of chlordiazepoxide should move to the neutral form, so the retention times of chlordiazepoxide should then increase.

Most retention times of the other substances tested have not been seriously affected by the changes in pH of the eluent. The retention times of over 100 selected substances, whose pK_a or pK_b values are not in the range between 3 and 6, had good reproducibility with an average CV% value of 2.09. But, the retention indices of the substances mentioned above had better reproduciblity with the CV% value of 1.57. The largest CV% value (22.5) expressed as retention times under the three pH values, decreased to 13.0% when the retentions under the same conditions were described as retention indices.

Effect of the Flow Rates

The aim of our test being to investigate the effect of the flowrate on the retention index, we chromatographed all the substances with flowrates of 0.9, 1.0 and 1.1 mL/min. Other conditions remained the same.

The retention times of 1-nitroalkane changed regularly and greatly under the three different flowrates. The results are given in Table 7. The retention times of the 115 tested substances also changed greatly under the same conditions. The average of the CV% values of the tested substances in retention times was 3.015, with a standard deviation of 0.817. Meanwhile, the average of the CV% values of the same substances is 1.143 with a standard deviation of 0.763.

It is well known that the flowrate of the eluent affects the retention times in HPLC. The greater the flowrate, the more quickly are the substances eluted. There is a simple relationship between the capacity factor k' and other chromatographic parameters:[25]

$k' = (\text{constant}) t_G * F/(\%B * Vm)$, where

%B = (%Acetonitrile at the beginning) – (%Acetonitrile at the end of the gradient elution);

F = Flowrate, in mL/min;

Vm = Volume of the column used;

t_G = gradient time.

There is a linear proportionality between flow rate and the capacity factor. Flow rate affects the retention times of 1-nitroalkane and the tested substances in a similar manner. That is why retention indices counteract the effect of flow rate on the reproducibility of the retention expression.

CONCLUSION

When the operating system, including pump, gradient profile, buffer, etc., is stable, the retentions, expressed both in terms of retention times and retention indices, in gradient HPLC, are well reproducible.

Retention index is only a method of linear correction. When the retention times of the analytes change proportional to that of the scale substances, the retention indices can well balance the variation from retention times. Some chromatographic conditions, such as column length, flow rate, gradient profile, etc., affect retention times greatly, meanwhile retention indices can decrease the effect and improve the reproducibility of the retention expression.

By contrast, some chromatographic conditions, for example, pH values of the buffer, etc., affect both retention times and retention indices of some substances because the retention times of the analytes and that of the retention index scale – 1-nitroalkane – do not change in the same way. However, the retention index method can somewhat improve the reproducibility of HPLC retention data.

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Received November 15, 1995
Accepted December 10, 1995
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J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1993–2007 (1996)

HPLC METHOD DEVELOPMENT FOR DULOXETINE HYDROCHLORIDE USING A COMBINATION OF COMPUTER-BASED SOLVENT STRENGTH OPTIMIZATION AND SOLVENT SELECTIVITY MIXTURE DESIGN

Bernard A. Olsen, Mark D. Argentine

Lilly Research Laboratories
Eli Lilly and Company
P. O. Box 685, Drop Code TL12
Lafayette, Indiana USA 47902

ABSTRACT

Computer simulation software for solvent strength optimization and statistical mixture design based on the solvent selectivity triangle were useful tools employed for the development of a reversed-phase HPLC method to separate duloxetine, a new anti-depressant compound, and structurally-related impurities. Solvent strength optimization was used to show that adequate separation for all impurities could not be obtained with a single organic modifier and to aid in choosing appropriate boundary conditions for a mixture design study. The mixture design was used to obtain resolution maps for organic modifier mixtures consisting of acetonitrile, methanol, and tetrahydrofuran. Overlapping resolution maps for the peak pairs of interest revealed the solvent composition that would provide the maximum resolution. Finally, solvent strength was optimized at the best solvent composition and information about method robustness obtained.

INTRODUCTION

Many computer-aided techniques for the development and optimization of high performance liquid chromatographic (HPLC) methods have been described.[1-8] Two of the more successful and widely-employed methods are solvent strength optimization using computer simulation,[9-14] and solvent selectivity optimization using a statistical mixture design.[15-26] With the solvent strength optimization technique, isocratic separations at various solvent strengths can be simulated after obtaining data from two gradient runs with different gradient slopes.

A disadvantage of this method is that only selectivity advantages derived from different solvent strengths are obtained. Different organic modifiers, or mixtures of modifiers, must each be treated as separate optimization experiments. No predictions are available for modifier combinations that have not been tested. This disadvantage, however, is the strength of solvent selectivity optimization using a mixture design approach.[15] With this technique, seven experiments from a statistical mixture design are performed using three different organic modifiers such as acetonitrile (ACN), methanol (MeOH), and tetrahydrofuran (THF).

Capacity factor and/or resolution data may then be mapped for any combination of organic modifiers and the optimum isocratic conditions chosen. The disadvantage of this technique is that no information about the separation at different solvent strengths other than those bounded by the experiment is obtained.[19] Also, a poor initial choice of boundary conditions for the mixture design can lead to suboptimal results.

In this paper, a combination approach to HPLC method development taking advantage of the complementary strengths of the solvent strength and mixture design selectivity techniques is described. The method development problem involved the separation of process-related impurities and degradation products in duloxetine hydrochloride, a serotonin/norepinephrine reuptake inhibitor currently undergoing clinical trials for the treatment of depression and urinary incontinence.

Structures of duloxetine and potential impurities are given in Figure 1. Compounds 4 and 5 are potential impurities from the synthetic process while 2, 3, and 6 are degradation products. Compounds 2 and 3 result from cleavage of the naphthyl ether and rearrangement to give the substituted naphthols. The initial cleavage products containing only the thiophene ring and aliphatic side chain were well-separated early in the chromatograms and were not included in the optimization.

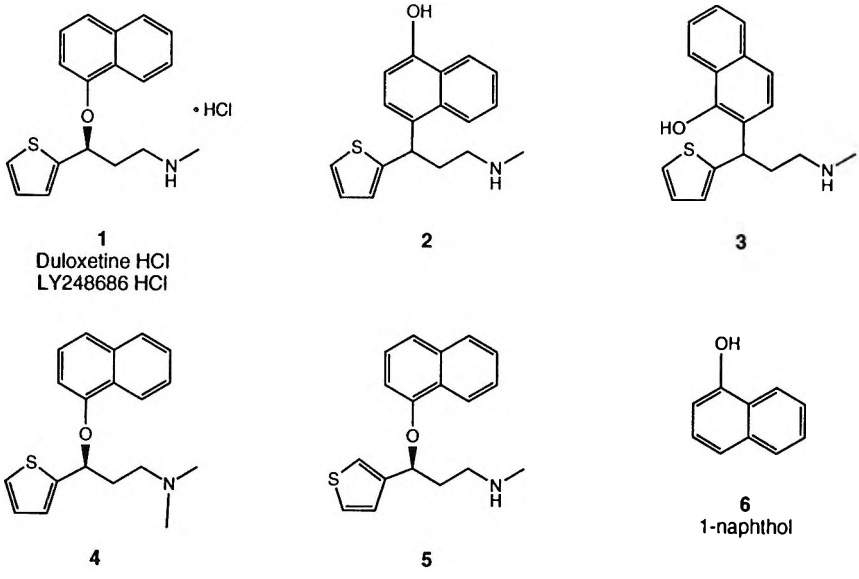

Figure 1. Duloxetine and potential impurities

- Perform two gradient runs with each organic modifier and simulate resolution vs. solvent strength
- For each modifier choose the optimum solvent strength from simulation results
- Perform 7-experiment mixture design study
- Generate resolution maps for peaks of interest
- Choose optimum solvent composition considering resolution and run time
- Perform two gradient runs using chosen modifier ratio
- Check ruggedness of separation and opportunities for optimization using solvent strength simulation

Figure 2. Combination HPLC method development approach employing solvent strength optimization and solvent selectivity mixture design.

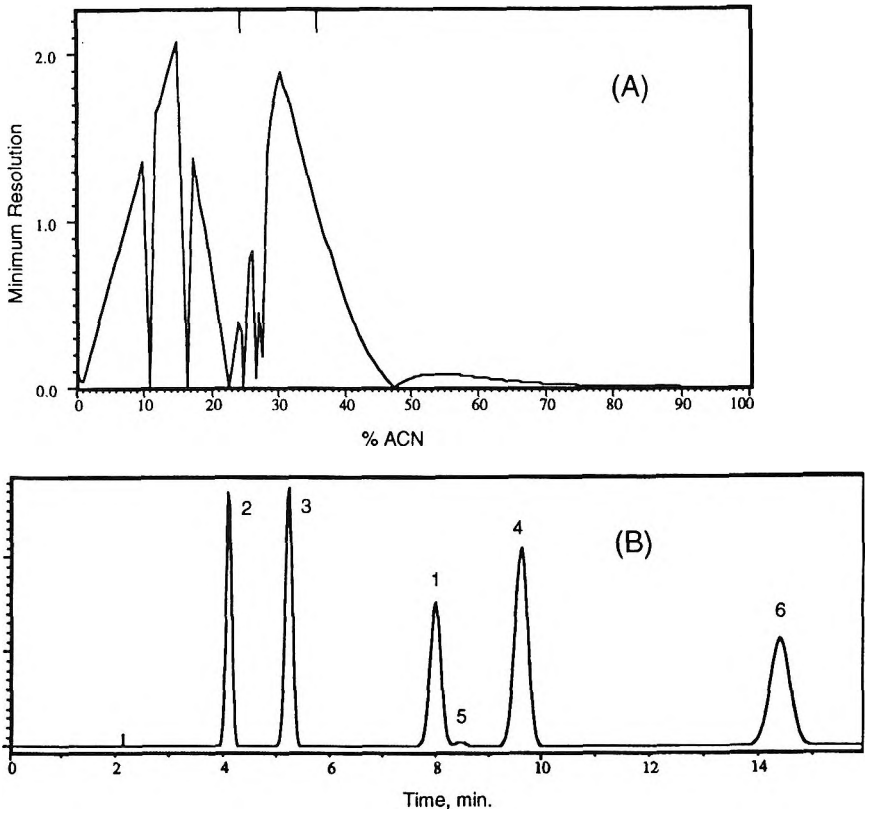

Figure 3. A) Resolution map for ACN modifier obtained using gradients from 20-50% ACN with gradient times of 20 and 40 minutes. Compounds 1 and 5 form the critical peak pair between 30 and 48% ACN. B) Chromatogram predicted for 35% ACN.

EXPERIMENTAL

Reagents

HPLC-grade acetonitrile, methanol, and tetrahydrofuran were obtained from EM Science (Gibbstown, NJ, USA). The mobile phase buffer was 50 mM potassium phosphate, pH 2.5, prepared using appropriate concentrations of potassium phosphate monobasic (EM Science), and orthophosphoric acid (85%, Fisher Scientific, Co., Fair Lawn, NJ, USA). The sample solvent was 30%

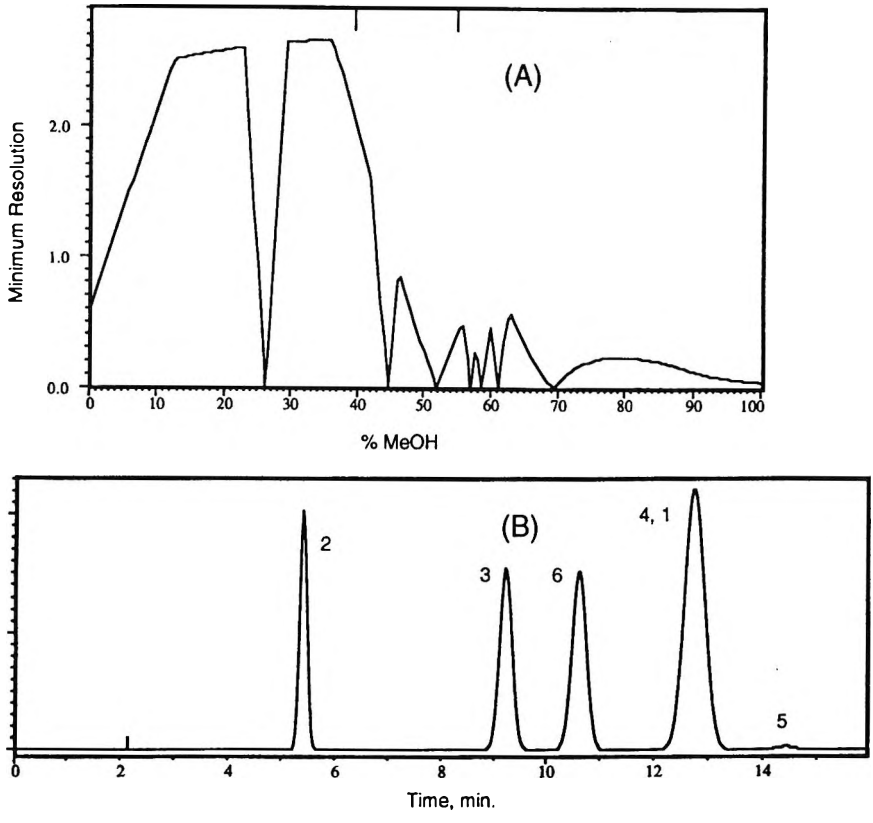

Figure 4. A) Resolution map for MeOH modifier obtained using gradients from 40-80% MeOH with gradient times of 20 and 40 minutes. Compounds 1 and 4 form the critical peak pair between 46 and 52% MeOH. B) Chromatogram predicted for 50% MeOH.

methanol in water. Water for mobile phases and sample solutions was purified with a Milli-Q system (Millipore, Milford, MA, USA). All mobile phase compositions are reported as volume/volume percentages of the aqueous buffer and organic modifiers.

Duloxetine hydrochloride and compounds 2-5 were from Lilly Research Laboratories. Compound 6, 1-naphthol, was obtained from Aldrich (Milwaukee, WI, USA) and recrystallized. Alternatively, compounds 2, 3, and 6 may be generated in solution by degrading duloxetine under acidic

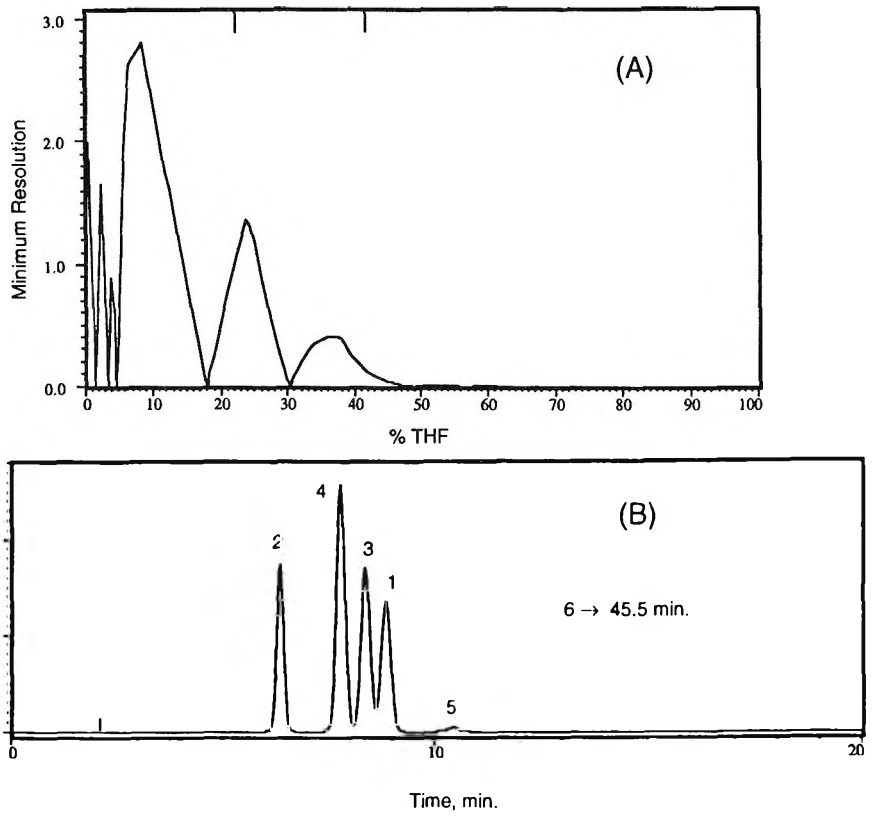

Figure 5. A) Resolution map for THF modifier obtained using gradients from 20-70% THF with gradient times of 20 and 40 minutes. Compounds 3 and 4 form the critical peak pair between 18 and 24% THF. Compounds 1 and 3 form the critical peak pair between 24 and 30% THF. B) Chromatogram predicted for 25% THF.

conditions. For example, a mixture of the degradation products was immediately formed upon addition of 0.1% v/v concentrated hydrochloric acid to a 0.1 mg/mL aqueous solution of duloxetine hydrochloride.

Apparatus and Conditions

The chromatographic system consisted of a Model 600 pump with column heater (Waters, Bedford, MA, USA), a Model 728 autoinjector (Alcott, Norcross, GA, USA) with a fixed-loop (10 μL) injection valve (Valco, Houston,

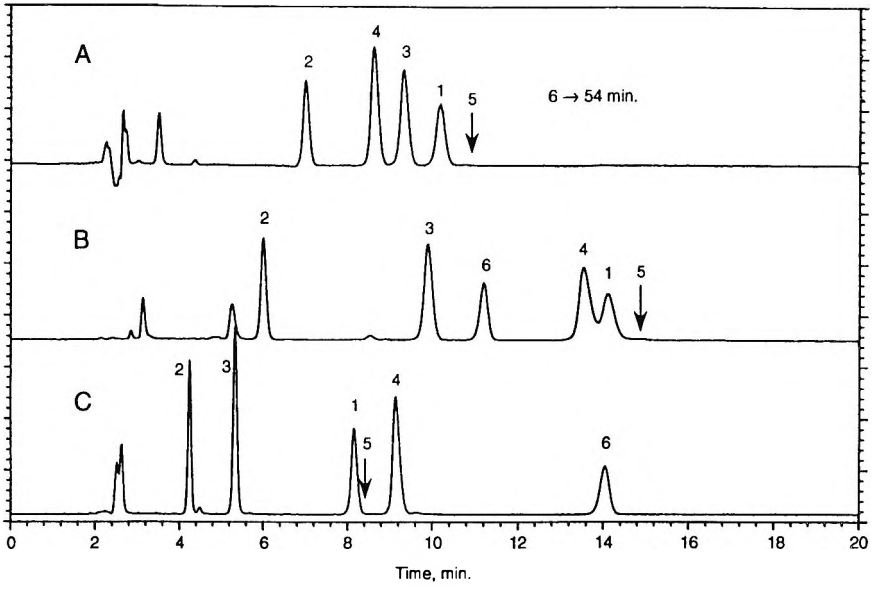

Figure 6. Chromatograms at vertex points of mixture design. A = 25% THF, B = 50% MeOH, C = 35% ACN. Retention of compound 5 indicated by arrows.

TX, USA), and a Model 787 variable wavelength UV detector set at 230 nm (Applied Biosystems, Ramsey, NJ, USA). Chromatograms were recorded using an in-house data acquisition system. A 250 mm x 4.6 mm ID, 5 μm particle size Zorbax RX-C_8 column (Mac-Mod Analytical, Chadds Ford, PA, USA) maintained at 35°C was used. The flow rate was 1.0 mL/min.

Software

DryLab G® software (version 1.53, LC Resources, Lafayette, CA, USA) was used for solvent strength optimization by calculating resolution versus solvent strength with data from two gradient runs for a given organic solvent. The statistical mixture design data were analyzed and resolution maps plotted with the JMP statistical software package (version 2.05 for the Macintosh, SAS Institute Inc., Cary, NC, USA). The overlapping resolution map was generated with a program written in QuickBASIC and plotted using Excel (Microsoft, Redmond, WA, USA).

RESULTS AND DISCUSSION

The combination development approach utilized in this study is outlined in Figure 2. Two gradient chromatograms for solvent strength studies are performed with each organic modifier: ACN, MeOH, and THF. Solvent strength optimization may show that one modifier will provide adequate results and no further development is needed. Results from these studies can also show whether selectivity changes using different modifiers warrant the use of a mixture design study and, if so, can aid in the choice of the individual modifier solvent strengths. If the retention order of peaks and their resolution values are relatively consistent for each modifier, solvent selectivity optimization may not be fruitful. If that is the case, pH optimization, other stationary phases, or modifiers such as ion-pairing reagents might be explored.

A low pH, where both the analytes and residual silanols on the stationary phase are protonated, was chosen for this study. Higher pH values might provide different selectivity but the separation may not be as rugged. Solvent boundary conditions can be chosen from the initial computer simulation results, and the mixture design is then conducted. Key peak resolutions are mapped to determine the optimum mobile phase composition for resolution. Optimization of analysis time may also influence the final choice of organic modifier conditions. Finally, the conditions chosen are then investigated for additional optimization and ruggedness using solvent strength modeling.

Retention data for duloxetine and impurities were obtained for two gradient runs using each organic modifier. Minimum resolution maps predicted by computer simulation are shown in Figures 3-5. Predicted isocratic chromatograms for each solvent at roughly equivalent solvent strengths are also shown. From these results, it was clear that relative peak retention varied greatly depending on the modifier used.

For example, the retention order of 4 and 1 was reversed between ACN and THF, while the peaks were coeluted with methanol at a comparable solvent strength. Also, 6 eluted before 1 with MeOH but after 1 with ACN, and it was very strongly retained (t_r = 45.5 minutes) with THF. The resolution between 1 and 5 with ACN was not sufficient to allow detection of small quantities of 5 (down to 0.1%) in the presence of 1 as the main component. Resolution of all peaks of interest was adequate with THF but the retention time of 6 was excessive.

The lack of acceptable results with a single modifier plus the significant differences in selectivity among the modifiers indicated that a solvent selectivity optimization should be performed. The following percentages of

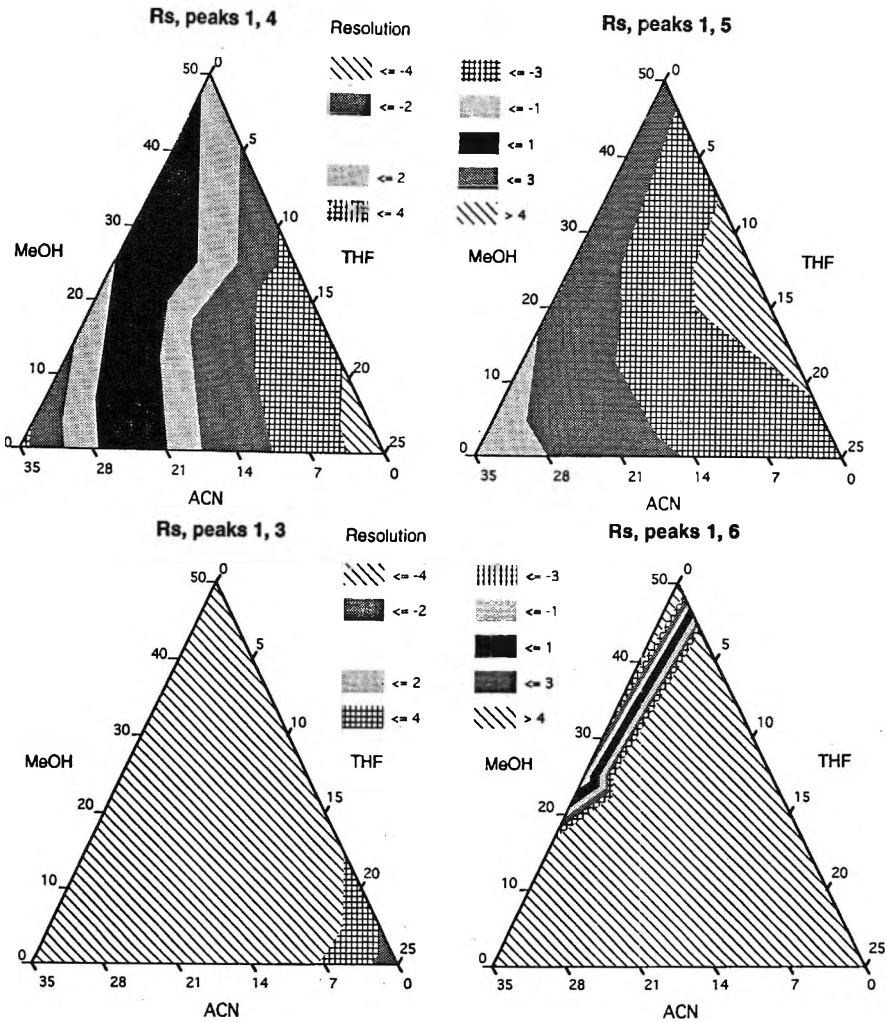

Figure 7. Resolution maps from solvent selectivity mixture design study.

each modifier were chosen for the mixture design study: ACN-35%, MeOH-50%, THF-25%. Although the percentages of ACN and MeOH were not those predicted to give maximum resolution, they allowed run times of less than 20 minutes without greatly compromising the resolution that was obtainable. It was not possible to maintain a reasonable resolution using only THF while keeping the run time under 20 minutes because of the long retention of 6.

Table 1

Solvent Selectivity Mixture Design Results

% Modifier			Resolution from Duloxetine[1]			
MeOH	ACN	THF	5	4	3	6
50	0	0	2.7	-1.2	9.6	-6.6
0	35	0	1.4	3.3	11.9	17.0
0	0	25	3.6	-4.5	2.5	33.4
25	17.5	0	2.3	1.0	12.5	-2.3
25	0	12.5	4.8	-3.4	5.1	36.8
0	17.5	12.5	2.9	-2.0	6.0	29.4
16.7	11.7	8.3	3.5	-2.4	7.6	17.6

[1]Resolution values are indicated as negative if impurity k′ is less than k′ of duloxetine.

The mixture design and resolution data from it are shown in Table 1. Resolution of impurities 3, 4, 5, and 6 from duloxetine were viewed as the key responses. Figure 6 shows chromatograms using single modifiers which correspond to the vertex points of the solvent selectivity triangle. The results agreed well with those predicted by simulation (Figures 3-5). Resolution maps for the four individual impurities from duloxetine, 1, are shown in Figure 7. Compound 3 was well-resolved under all conditions and 6 had only a narrow band of conditions producing poor resolution. Compound 5 had the lowest resolution from 1 in the region of high ACN modifier content, while compound 4 was not resolved over a significant portion of the selectivity map.

The best resolution conditions appeared to be toward the THF/MeOH axis and away from ACN. This was confirmed by an overlapping resolution map showing the minimum resolution for all four peak pairs over the range of solvent composition (Figure 8).

The following mobile phase composition was predicted to give a minimum resolution of 4.0: 2.5% ACN, 11% MeOH, 17.5% THF, and 69% buffer. While providing optimum resolution, the relatively high percentage of THF led to excessive retention of 6. Since MeOH provided decreased retention

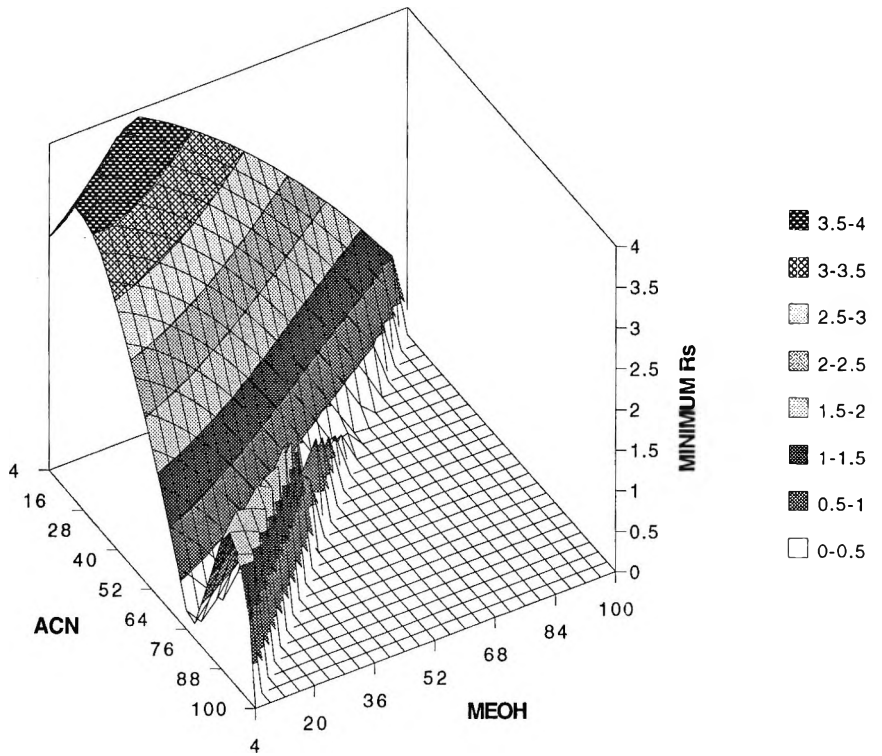

Figure 8. Overlapping resolution map from solvent selectivity mixture design study. Absolute MeOH and ACN percentages can be found by multiplying by 0.50 and 0.35, respectively. THF percentage can be found by subtracting ACN and MeOH (on graph) from 100 and multiplying by 0.25.

of 6 relative to other components, resolution predictions were obtained at increased MeOH concentrations. Also, ACN was eliminated to simplify the mobile phase. A composition of 25% MeOH, 12.5% THF was predicted to give a minimum resolution of 3.4 versus the optimum value of 4.0.

The run time was reduced even further by modifying the composition to 35% MeOH, 10% THF. This relative modifier ratio (35:10) was then used for final solvent strength optimization. Alternatively, a chromatographic response function such as that employed by Glajch et al. could have been used to simultaneously evaluate both run time and resolution during the solvent selectivity mixture design.[15]

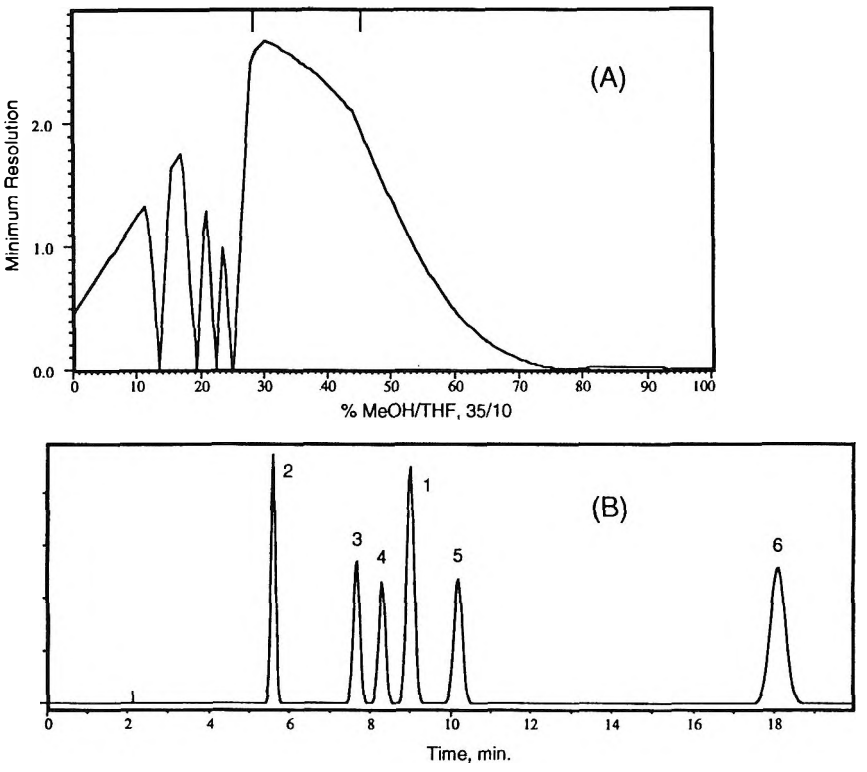

Figure 9. A) Solvent strength resolution map for MeOH/THF modifier obtained using gradients from 20-70% MeOH/THF, 35/10 with gradient times of 30 and 60 minutes. Compounds 1 and 4 form the critical peak pair between 30 and 44% MeOH/THF. Compounds 3 and 4 form the critical peak pair between 44 and 77% MeOH/THF. B) Chromatogram predicted for 45% MeOH/THF, 35/10.

Two gradient runs were performed using a mixture of MeOH and THF, 35/10, as the organic modifier. Figure 9 shows the minimum resolution map for this study. A simulated isocratic chromatogram at 45% of the MeOH/THF mixture (which corresponds to an overall mobile phase composition of 35% MeOH, 10% THF, and 55% buffer) shows greater than baseline resolution for all peaks from duloxetine with a run time of about 19 minutes.

Also, the resolution map is not steeply sloping over the solvent range of interest, indicating that the separation should be fairly rugged toward small

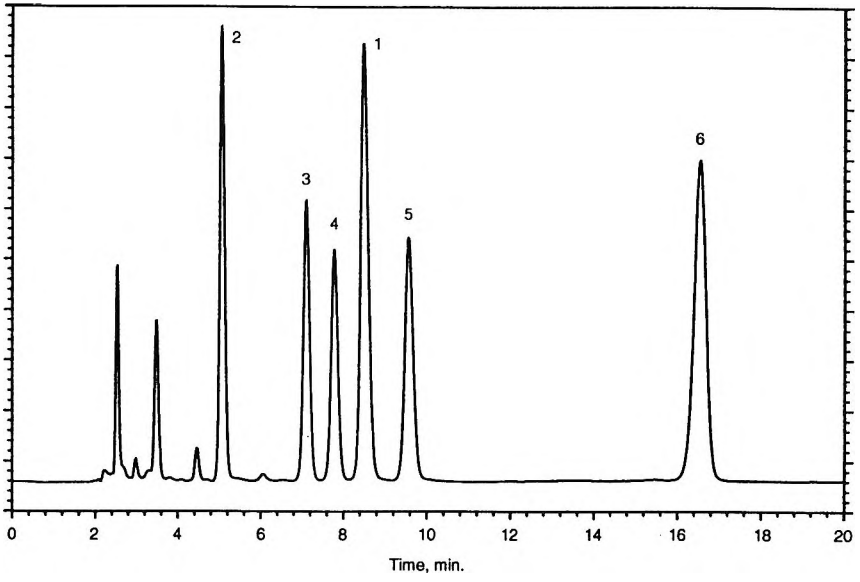

Figure 10. Experimental chromatogram at 44% MeOH/THF, 35/10.

changes in mobile phase composition. Slightly greater resolution could be obtained, if needed, by decreasing the MeOH/THF concentration, although the run time would lengthen. A chromatogram obtained using 44% of the MeOH/THF mixture is shown in Figure 10. Experimental retention times were about 10% less than those from the simulation which is within the agreement expected considering the accuracy of the simulation and mobile phase mixing.

CONCLUSIONS

The combination approach to mobile phase optimization provided rugged conditions which gave an acceptable separation of duloxetine from related impurities in under 20 minutes. In addition to indicating the mobile phase composition for optimal separation, the resolution maps from solvent strength simulations and the mixture design technique provide information about the separation ruggedness.

This information can also be used to adjust conditions appropriately to compensate for column or instrumental differences that may be encountered in the future.

ACKNOWLEDGMENTS

Technical assistance from Ms. D. Harper and information from Mr. M. Skibic and Mr. W. Smith are gratefully acknowledged.

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Received October 9, 1995
Accepted January 2, 1996
Manuscript 3968

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 2009–2024 (1996)

LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS DETERMINATION OF PARAQUAT AND DIQUAT IN PLASMA, URINE AND VITREOUS HUMOUR

I. Sánchez Sellero, A. Cruz, P. Fernández,
M. López-Rivadulla*

Service of Forensic Toxicology
Institute of Legal Medicine
Faculty of Medicine
University of Santiago de Compostela
C/ San Francisco s/n
15705 Santiago de Compostela (La Coruña), Spain

ABSTRACT

An HPLC method for the simultaneous determination of paraquat and diquat in aqueous solutions and biological fluids (plasma, urine and vitreous humour) was developed. This method is based on the initial ion-pair solvent extraction of both herbicides from plasma or urine. Vitreous humour samples required a protein precipitation and concentration process. Relatively small sample volumes (1 mL of plasma or urine, and 100 µL of vitreous humour) were enough to determine paraquat and diquat by the proposed technique. Chromatography was carried out using a LiChrospher® 100 RP-18 (5 µm) column for aqueous solutions and plasma and urine extracts, or a Nova-Pak C_{18} (3.9x150 mm) column for vitreous humour extracts. Two ultraviolet wavelengths were selected, 254 nm for paraquat and 310 nm for diquat. The calibration curves were linear in the concentration ranges 0.42-8.4 µg/mL for aqueous solutions, 0.1-2 µg/mL for plasma, 0.1-3 µg/mL for urine and 0.5-5 µg/mL for vitreous humour.

INTRODUCTION

Weeds have been an important trouble in agriculture since the far-distant past. Some attempts for their control have been assumed through the ages. Nevertheless, the use of chemicals caused a real revolution in the weed control after World War II.

Bipyridylium herbicides (paraquat and diquat) are widely used in agriculture. Paraquat, prototype of that group, was first commercialized in 1962. The safety of paraquat in its correct use is well known. However, serious poisoning and high mortality have been associated with accidental or suicidal ingestion of paraquat. Although various measures to prevent paraquat poisoning (such as the addition of emetics, dyes, odorants and bitter substances) have been introduced, high lethality of paraquat hasn't been reduced. Since diquat toxicity is lower than paraquat toxicity, it has been considered advisable to reduce paraquat content of commercial product and to replace it with diquat.

Determination of paraquat (PQ) and diquat (DQ) is required to know if these compounds are involved in a poisoning, to assess the severity of the intoxication,[1-5] and to monitor the terapy.

Spectrophotometric determination of PQ after reduction with dithionite is probably the most used technique. Derivative spectroscopy methods (6-8) have been recently reported for the determination of PQ and DQ in biological fluids. The use of derivative mode enhances the sensitivity and specificity of spectroscopy, as interference is eliminated. Other techniques, such as RIA,[9-12] PFIA,[13] ELISA,[14-16] GC,[17-19] TLC-FID[20] or capillary electrophoresis,[21] have been proposed. RIA and PFIA are very sensitive techniques and require small sample volumes, but cannot be commonly used.

Gill et al.[22] described in 1983 a method for the determination of both PQ and DQ in urine. The reported lower limit of detection was approximately 1 μg/mL for both herbicides. That limit required to be improved.

Querée et al.[23] reported in 1985 an HPLC method for the determination of PQ in liver and haemolysed blood which was based on the ion-pair solvent extraction of PQ with sodium dodecyl sulphate prior to ion-pair reverse phase chromatography. The reported limit of detection for haemolysed blood was 0.05 μg/mL.

Nakagiri et al.[24] developed in 1989 a new system in which an automated pretreatment apparatus was connected to ion-exchange HPLC. Measurement of PQ and DQ was automatically carried out after injecting a microsample of serum or urine into an injection port.

An ion-pair, reverse phase HPLC method with ultraviolet (UV) detection was later developed by Corasaniti et al.[25] to measure PQ concentrations in brain.

Ito et al.[26] have recently proposed a liquid chromatographic method for simultaneous determination of PQ and DQ in human tissues. This method was based on the extraction of PQ and DQ from the sample using a Sep-Pak C_{18} cartridge. L-tyrosine was used as the internal standard.

Chromatography was carried out using an octadecyl silica column with a mobile phase of potassium bromide in methanol solution. Two UV wavelengths were selected, 256 nm for PQ as well as the internal standard, and 310 nm for DQ. The lower limit of detection was 0.05 µg/g.

Other authors[27,28] developed liquid chromatographic methods for determination of PQ and DQ in crops.

Gill's HPLC method for the quantification of PQ in urine has been applied to serum by Croes et al.[29] Sample preparation consisted of ion-pair extraction on disposable cartridges of end-capped octadecyl silica. PQ was determined by HPLC using 1,1'-diethyl-4,4'-dipyridyl dichloride as an internal standard.

A reverse phase ion-pair high performance liquid chromatographic system with UV detection is presented. This method separates PQ and DQ in under 8 min.

A rapid procedure for the previous extraction of the herbicides from plasma and urine, has been tuned up. The extraction method is a modified version of one reported by Querée et al.[23] Pretreatment of vitreous humour samples consisted of a simple protein precipitation and sample concentration process.

MATERIAL

Reagents

Chemicals and HPLC-grade solvents were obtained from Merck. So, methanol, orthophosphoric acid, sulphuric acid, sodium dihydrogenphosphate, n-hexane, acetonitrile, diethylamine, methylisobutylketone (MIBK), isobutanol and sodium dodecyl sulphate were used. Heptanesulphonic acid sodium salt and octane sulphonic acid were supplied by Sigma, and sodium carbonate by Panreac. All chemicals used were of analytical grade.

Standards

Paraquat dichloride was obtained from Sugelabor (Barcelona). Diquat dibromide was purchased from ICI.

Apparatus

Chromatograms were obtained from a high-performance liquid chromatographic system consisting of a manual injector, LiChrospher® 100RP-18 (5 μm) column in LiChroCart® 125-4 (Merck) preceded by a 4x4 mm guard column (C_{18} reversed-phase, particle size 5 μm), or 3.9x150 mm Waters Nova-Pak C_{18} column with Waters Guard-Pak™ precolumn for vitreous humour samples, two Waters Model 501 pumps, a Waters Model 490 programmable multiwavelength UV detector, and a Waters system interface module. That chromatographic system was interfaced to a NEC PowerMate SX/16 microcomputer running MAXIMA&BASELINE software.

METHODS

Solvents and Reagents Preparation

The MIBK was washed with 100 mL sodium carbonate (100 g/L) per liter of MIBK and then with distilled water. The extractant was prepared by mixing equal volumes of water-saturated isobutanol and MIBK, into which enough

sodium n-dodecylsulphate was dissolved to obtain a final concentration of 25 g/L (23). 1M aqueous sulphuric acid was used as the aqueous phase of the plasma and urine extraction procedure. 0.5 and 2M H_2SO_4 were also tested.

Preparation of Paraquat and Diquat Solutions

PQ dichloride was dried to constant weight at 100°C overnight and stored in a dessicator prior to use. Two stock solutions containing 50 mg/L and 100 mg/L, were then prepared in redistilled water.

Standards of DQ dibromide containing 50 mg/L and 100 mg/L, were also made similarly in redistilled water.

The above stock solutions were used to make working solutions containing 0.42-8.4 μg/mL PQ and/or DQ in mobile phase. The concentration range

Table 1

Working Solutions

Set	Matrix	Concentrations (μg/mL) PQ	DQ
PQ	Aqueous	0.42-2.1-4.2-6.3 and 8.4	----------
	Plasma	0.1-0.5-1.0-1.5 and 2.0	----------
	Urine	0.1-0.5-1.0-2.0 and 3.0	----------
	Vitreous Humour	0.5-1.0-2.0-3.0 and 5.0	----------
DQ	Aqueous	----------	0.42-2.1-4.2-6.3 and 8.4
	Plasma	----------	0.1-0.5-1.0-1.5 and 2.0
	Urine	----------	0.1-0.5-1.0-2.0 and 3.0
	Vitreous Humour	----------	0.5-1.0-2.0-3.0 and 5.0
PQ + DQ	Aqueous	0.42-2.1-4.2-6.3 and 8.4	0.42-2.1-4.2-6.3 and 8.4
	Plasma	0.1-0.5-1.0-1.5 and 2.0	0.1-0.5-1.0-1.5 and 2.0
	Urine	0.1-0.5-1.0-2.0 and 3.0	0.1-0.5-1.0-2.0 and 3.0
	Vitreous Humour	0.5-1.0-2.0-3.0 and 5.0	0.5-1.0-2.0-3.0 and 5.0

studied in plasma was 0.1-2 μg/mL for both compounds. Although the mentioned ranges for aqueous and plasma solutions are apparently different, the net amounts of each compound from aqueous solutions injected onto the HPLC, are the same as those from plasma solutions if the recovery obtained with the extraction procedure applied was 100%.

To calculate the recovery of the extraction method, we must consider that not only 4.2 mL of the 5 mL of extraction solvent used are transferred to a clean tube, but also 25 μL of the 200 μL H_2SO_4 added at the end of the process are injected onto the HPLC.

The concentration ranges selected for urine and vitreous humour samples were 0.1-3 μg/mL and 0.5-5 μg/mL, respectively.

We assayed three types of solutions, namely: a) a set containing PQ, b) another set containing DQ, and c) mixtures of the two pesticides, as can be seen in table 1. All these solutions were prepared in triplicate

Extraction Procedure

The extraction procedure used was based on one reported by Querée et al.[23] Nevertheless, our liquid-liquid extraction method is shorter and easier to perform than the method of Querée.

A volume of 1 mL of biological fluid (plasma or urine) containing a given concentration of one or the two herbicides was mixed gently with 1 mL of redistilled water and 5 mL of extractant on a roller mixer and then centrifuged for 10-15 min, after which 4,2 mL of the organic layer were extracted and added 200 µL of 1M H_2SO_4. The mixture was then shaken vigorously for 5 min and centrifuged for 2 min, after which the acid extract was recovered and washed twice with 2 mL of n-hexane. 25 µL of the washed extract were injected onto the HPLC.

Preparation of Vitreous Humour Samples

100 µL of vitreous humour and 100 µL of acetonitrile were mixed and shaken. The mixture was then centrifuged and 170 µL of the upper layer were recovered and concentrated to dryness into the speed-vac. The residues were dissolved in 28 µL mobile phase, 25 µL of which were injected onto the column.

HPLC Method

The analytical columns used were those previously reported. Guard columns were also used. After different mobile phases were assayed, one proposed by Querée et al.[23] was selected to be applied to our chromatographic study. This one consisted of 25% aqueous methanol containing 10mM octane sulphonic acid and 13.4 mL/L o-phosphoric acid. Diethylamine was used to adjust the pH of the mobile phase to 3. The flow rate was set at 1 mL/min. The injected volume was 25 µL. We examined the suitability of different wavelengths to detect PQ and DQ. In spite of the first tests with aqueous solutions of mixtures of PQ and DQ confirmed that both compounds can be detected at 290 nm, we selected two detection wavelengths, 254 nm for PQ and 310 nm for DQ. Detector AUFS was set as referenced below:

MATRIX	254 nm	310 nm
Aqueous	0.06 aufs	0.03 aufs
Plasma	0.03 aufs	0.03 aufs
Urine	0.03 aufs	0.03 aufs

The separation of PQ and DQ was complete within 8 min.

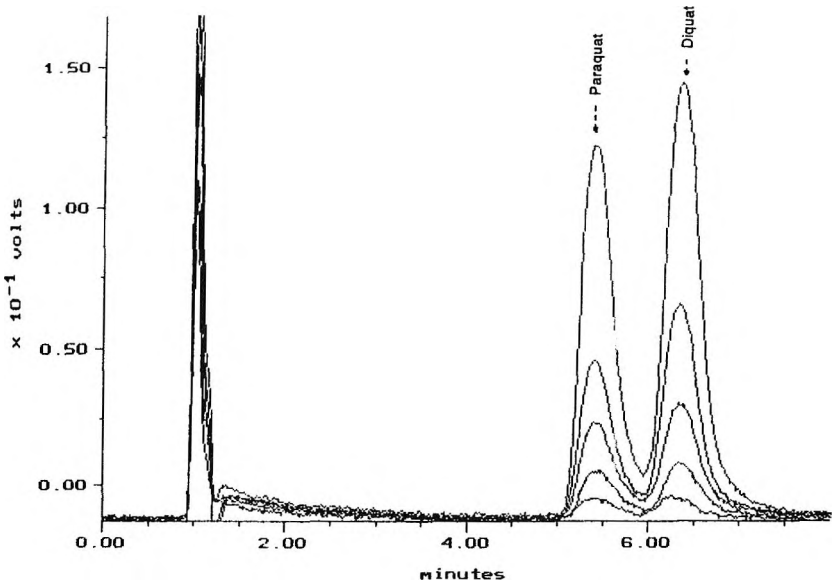

Figure 1. Chromatograms of mixtures of PQ and DQ in mobile phase at 290 nm.

RESULTS

Figure 1 shows the chromatograms of mixtures of PQ and DQ (concentration range 0.4-8 μg/mL for both compounds) obtained at 290 nm.

There is a linear relationship between the peak areas of each one of the two compounds and the amounts of these in the injection volume. Table 2 lists the results of the regression analysis performed.

As can be seen in figures 2, 3, 4 and 5, the chromatograms obtained under the selected conditions, at 254 and 310 nm (for the detection of PQ and DQ, respectively), show two peaks corresponding to as many compounds with retention times of 4.258 ± 0.4258 min (PQ, 254 nm) and 5.183 ± 0.5183 min (DQ, 310 nm).

The peak areas of PQ and DQ were found to be linearly related to the drug concentrations. Table 3 summarizes the results of the regression analysis performed.

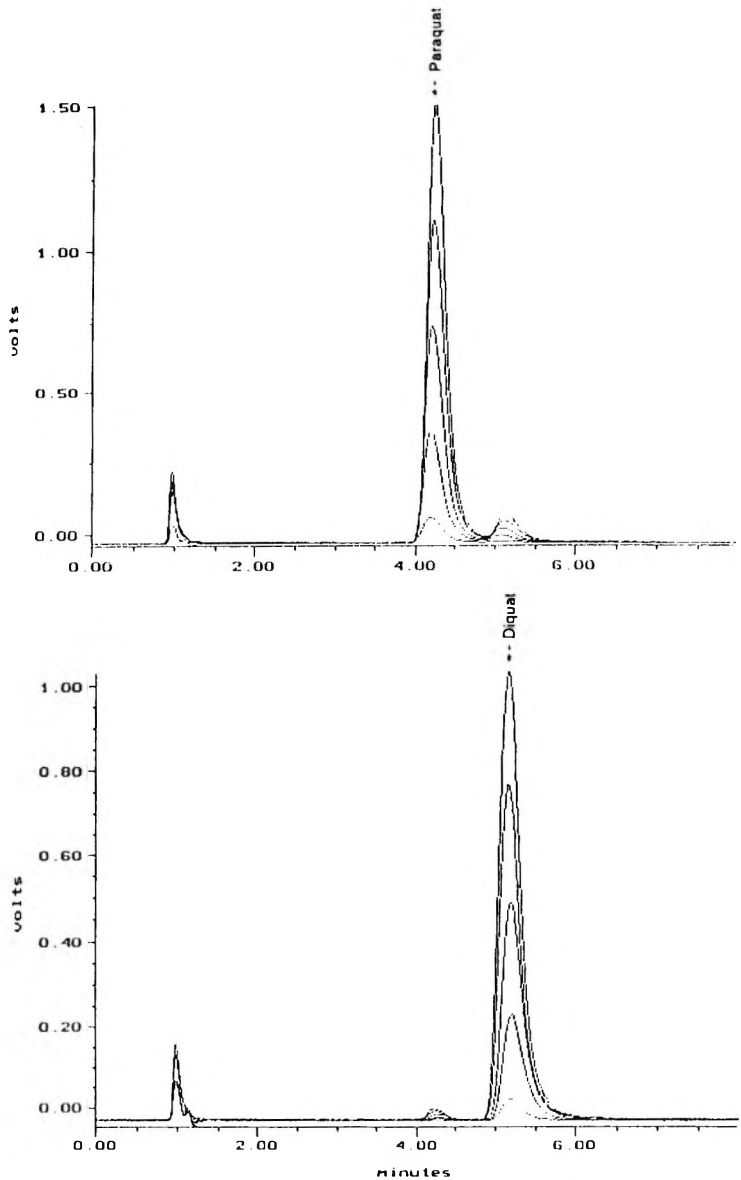

Figure 2. Chromatograms of mixtures of PQ and DQ in mobile phase at 254 nm (at the top) and 310 nm (at the bottom).

Table 2

Equations of the Calibration Curves Obtained for Paraquat and Duquat in mobile phase at 290 nm (n = 3)

Compound	Solution	Intercept	Slope	Correlation Coefficient
PQ	PQ (0.4-8 µg/mL) + DQ (0.4-8 µg/mL)	3.302E-02	1.458E-06	0.9975
DQ	PQ (0.4-8µg/mL) + DQ (0.4-8 µg/mL)	4.111E-02	1.22E-06	0.9991

$y = ax + b$
x = peak area
y = concentration injection volume

The mean recoveries of PQ from plasma samples, with or without DQ, were 36% and 38%, respectively. The recoveries of PQ from urine were 23% and 16%, respectively.

The average recoveries obtained for DQ in plasma were 34.5% and 53.5%, with or without PQ, respectively. The mean values in urine were 40% and 35%, respectively.

In spite of the above mentioned recoveries, a good sensitivity for both herbicides was achieved. The quantification limits were 0.1 µg of PQ or DQ per millilitre of plasma or urine, and 0.5 µg of PQ or DQ per millilitre of vitreous humour.

The reproducibility of our method was determined by analysing every solution shown in table 1 in triplicate. The average coefficients of variation for PQ in mobile phase, plasma, urine and vitreous humour were 5, 11, 8 and 5, respectively. The mean coefficients of DQ in these types of matrix were 2, 12, 6 and 5, respectively. These values can be considered acceptable.

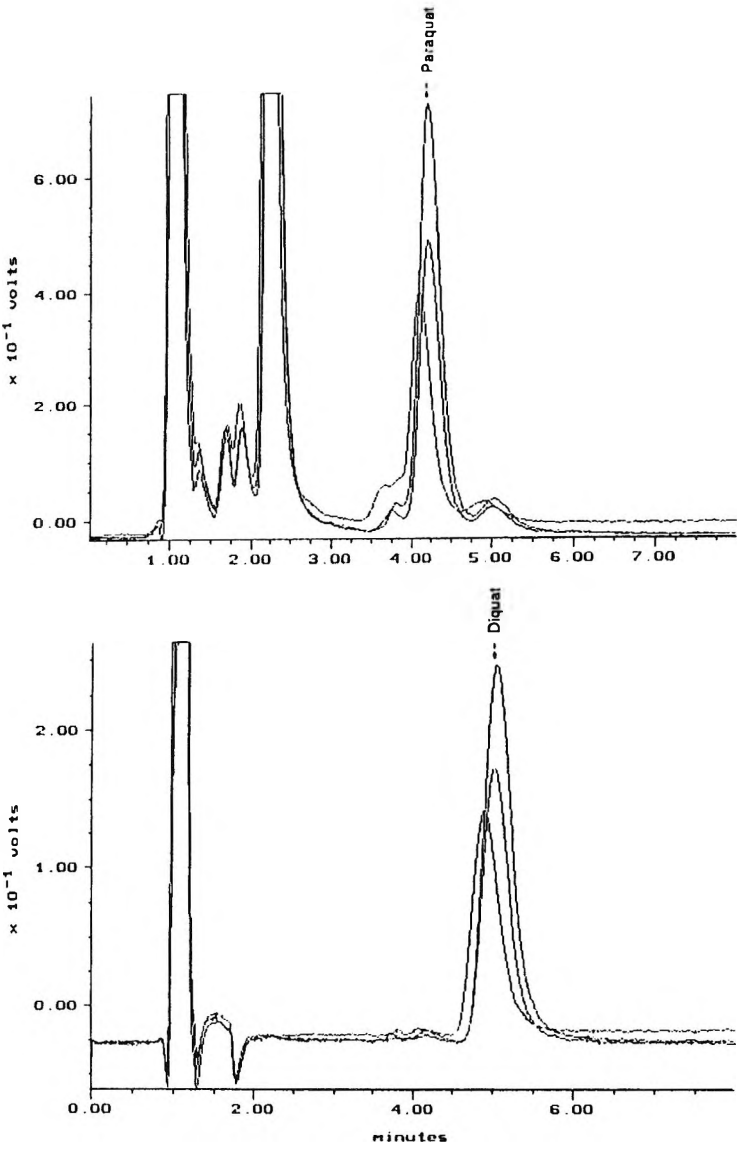

Figure 3. Chromatograms of mixtures of PQ and DQ in plasma at 254 nm (at the top) and 310 nm (at the bottom).

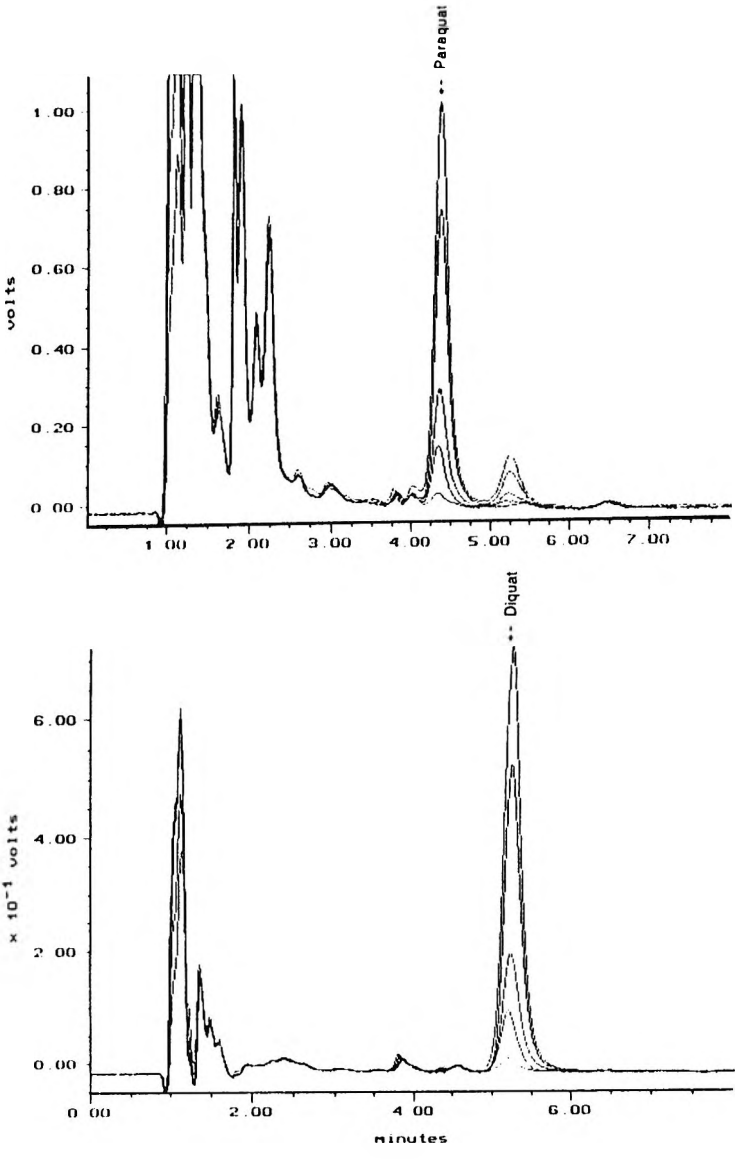

Figure 4. Chromatograms of mixtures of PQ and DQ in urine at 254 nm (at the top) and 310 nm (at the bottom).

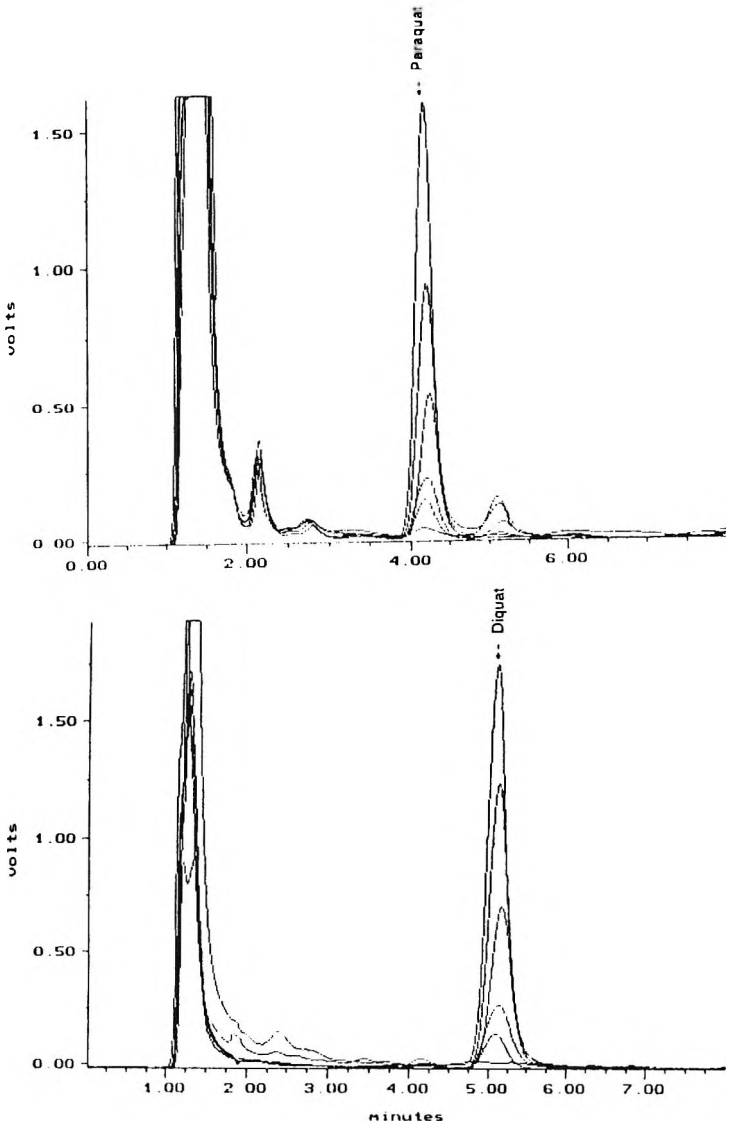

Figure 5. Chromatograms of mixtues of PQ and DQ in vitreous humour at 254 nm (at the top) and 310 nm (at the bottom)

Table 3

Equations of Calibration Curves Obtained for Paraquat and Diquat at 254 and 310 nm, respectively (n = 3)

Compound	Matrix	Solution	Intercept	Slope	Correlation Coefficient
PQ	Aqueous	PQ alone	-6.060E+05	23.9336E+06	0.9886
		PQ + DQ	-2.203E+05	24.6351E+06	0.9994
	Plasma	PQ alone	-5.454E+04	8.2598E+06	0.9898
		PQ +DQ	-1.254E+04	7.9721E+06	0.9810
	Urine	PQ alone	1.566E+04	4.3864E+06	0.9933
		PQ + DQ	-3.375E+05	4.4409E+06	0.9875
	Vitreous Humour	PQ alone	3.188E+05	4.7392E+06	0.9911
		PQ + DQ	-2.045E+05	4.5026E+06	0.9872
DQ	Aqueous	DQ alone	-0.395E+04	10.4855E+06	0.9996
		DQ + PQ	-11.499E+04	10.5110E+06	0.9997
	Plasma	DQ alone	-8.922E+04	3.8335E+06	0.9847
		DQ + PQ	3.325E+05	3.6115E+06	0.9700
	Urine	DQ alone	1.248E+05	3.9853E+06	0.9948
		DQ + PQ	-2.039E+05	4.229E+06	0.9922
	Vitreous Humour	DQ alone	-4.143E+04	6.9661E+06	0.9984
		DQ + PQ	1.087E+05	5.9756E+-06	0.9958

$y = ax + b$
x = concentration (μg/mL)
y = peak area

DISCUSSION

The proposed HPLC method separates PQ and DQ. Quantification of low levels of these compounds, with an important prognostic value, can be made by this method.

Despite the good results obtained with the first tests at 290 nm, we chose two wavelengths, 254 and 310 nm, to detect PQ and DQ, respectively. PQ and DQ peaks were successively recorded at 290 nm (figure 1). There was nearly 1 min between both peaks, but the tail of PQ peak overlapped the initial plot of DQ peak.

The absorptivity of PQ at 254 nm and the one of DQ at 310 nm were stronger than those at 290 nm. Thus the presence of PQ and DQ can be determined by selecting the appropriate detector wavelength, 254 nm for PQ

and 310 nm for DQ. This procedure enhanced the sensitivity of the method for both herbicides. Moreover, PQ absorptivity at 310 nm and DQ absorptivity at 254 nm were very low. So, the interference observed at 290 nm was extremely reduced.

The liquid-liquid ion-pair extraction method developed offers the advantage that is more expeditious and easier to perform than the original extraction procedure of Querée. In the case of vitreous humour samples, the features of this biological medium allow to apply a rapid and simple procedure consisting of protein denaturalization and precipitation following by a concentration process. This sample pretreatment is applied on a little volume of that one (100 µL), which is a very important characteristic to study fatal real cases of poisoning.

The quantification limits achieved (0.1 µg/mL of PQ or DQ in plasma or urine, and 0.5 µg/mL of PQ or DQ in vitreous humour) happened to be adequate at the sight of the results obtained in our laboratory, where cases of poisoning caused by PQ and DQ or PQ alone were studied. The developed chromatographic method has been also used by our group for determination of PQ and/or DQ concentrations in a toxicokinetic study of these compounds made in rabbits and not yet published.

ACKNOWLEDGEMENTS

This work was financially supported by the Dirección Xeral de Ordenación Universitaria of the Xunta de Galicia as part of Project XUGA 8421389.

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Received December 5, 1995
Accepted January 2, 1996
Manuscript 4045

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 2025 (1996)

ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

A Two-Day In-House Training Course

The course, which is offered for presentation at corporate laboratories, is aimed at chemists, engineers and technicians who use, or plan to use, high performance liquid chromatography in their work. The training covers HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC hardware module and system problems.

The following topics are covered in depth:

- Introduction to HPLC Theory
- Modes of HPLC Separation
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- Gradient Elution Techniques
- Calibration and Quantitation
- Logical HPLC System Troubleshooting

The instructor, Dr. Jack Cazes, is founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Series Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on Gel Permeation Chromatography for many years.

Details of this course may be obtained from Dr. Jack Cazes, P. O. Box 2180, Cherry Hill, NJ 08034-0162, USA. Tel: (609) 424-3505; FAX: (609) 751-8724; E-Mail: jcazes@voicenet.com.

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 2027–2037 (1996)

LIQUID CHROMATOGRAPHY CALENDAR

1996

JULY 14 - 18: 5th World Congress of Chemical Engineering, Marriott Hotel, San Diego, California. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

JULY 27 - 31: 37th Annual Meeting of the Aqmerican Society of Pharmacognosy, University of California, Santa Cruz, California. Contact: Dr. Roy Okuda, Chem Dept, San Jose State University, One Washington Square, San Jose, CA 95192-0101, USA. Tel: (408) 924-5000; FAX: (408) 924-4945.

AUGUST 7 - 9: 28th Canadian High Polymer Forum, Sarnia, Ontario, Canada. Contact: Kar Lok, BASF Corp, 11501 Steele Creek Rd, Charlotte, NC, 28273, USA. Tel: (704) 587-8240; FAX: (704) 587-8115.

AUGUST 8 - 10: 3rd Annual Symposium on Biomedical, Biopharmaceutical and Clinical Applications of Capillary Electrophoresis, Mayo Clinic, Rochester, Minnesota. Contact: Dr. S. Naylor, Mayo Foundation, Section of Continuing Education, Rochester, MN 55905, USA.

AUGUST 9 - 14: 31st Intersociety Energy Conversion Engineering Conference (co-sponsored with IEEE), Omni Shoreham Hotel, Washington, DC. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 11 - 15: 26th ACS Northeast Regional Meeting, Western Conn State Univ, Danbury, CT. Contact: A. Alder, 11 Long Ridge Rd, Redding, CT 06896, USA; (203) 938-2920; Email: reglmtgs@acs.org.

AUGUST 11 - 16: 3rd International Hydrocolloids Conference, Sydney, Australia. Contact: Gail Hawke, P. O. Box N-399, Grosvenor Place, Sydney, NSW 2000, Australia. Tel: 61 02 241 3388; FAX: 61 02 241 5282.

AUGUST 11 - 16: ICORS '96: 15th International Conference on Raman Spectroscopy, Pittsburg, Pennsylvania. Contact: Sanford Asher, Chem Dept, University of Pittsburgh, PA 15260, USA. Tel: (412) 624-8570.

AUGUST 12 - 16: 11th Internationa Congress on Thermal Analysis & Calorimetry, Philadelphia. Contact: The Complete Conference, 1540 River Pk Dr, Sacramento, CA 95815, USA. Tel: (916) 922-7032.

AUGUST 17 - 20: 31st National Heat Transfer Conference, Westin Galleria, Houston, Texas. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 18 - 23: 17th International Conference on Magnetic Resonance in Biological Systems, Keystone, Colorado, USA. Contact: Conference Office, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4735; FAX: (505) 989-1073.

AUGUST 21 - 23: 4th International Symposium on Capillary Electrophoresis, York, UK. Contact: Dr. T. Threlfall, Industrial Liaison Executive, Dept of Chem, University of York, Heslington, York, YO1 5DD, UK.

AUGUST 25 - 29: 212th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; Email: natlmtgs@acs,org.

AUGUST 25 - 30: International Symposium on Metal Hydrogen Systems: Fundamentals and Applications, Les Diablerets, Switzerland. Contact: MH-96, Inst of Physics, Univ of Fribourg, Perolles, CH-1700 Fribourg, Switzerland. Tel: 41 37 299 113; FAX: 41 37 299 772.

AUGUST 25 - 30: 12th International Congress on Chemical & Process Engineering, Praha, Czech Republic. Contact: Organizing Committe, CHISA'96, P. O. Box 857, 111 21 Praha, Czech Republic. Tel: 42 2 353287; FAX: 42 2 3116138.

SEPTEMBER 1 - 4: 4th Inetrnational Symposium on Preparative & Industrial Chromatography & Related Techniques, Basel, Switzerland. Contact: Secretariat Prep'96, Messeplatz 25, CH-4021 Basel, Switzerland. Tel: 41 61 686 28 28; FAX: 41 61 686 21 85.

SEPTEMBER 1 - 6: IUPAC Chemrawn IX, Seoul, Korea. Contact: IUPAC Chemrawn IX, Secretariat, Tongwon B/D 6th Floor, 128-27 Tangjudong, Chongro-ku, Seoul 110-071, Korea. FAX: 82 2 739-6187.

SEPTEMBER 1 - 6: 11th International Symposium on Organosilicon Chemistry, Monpellier, France. Contact: R. Corriu, University of Montpellier II, Place Eugene Batallon, cc007, 34095 Montpellier cedex 05, France. Tel: 67 14 38 01.

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization," Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

SEPTEMBER 4 - 9: International "Thermophiles '96" Symposium: Biology, Ecology & Biotechnology of Thermophilic Organisms, Athens, Georgia. Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; FAX: (706) 542-2651.

SPETEMBER 7: Field-Flow Fractionation Workshop VIII, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy. Tel: 39 532 291154; FAX: 39 532 240709.

SEPTEMBER 9 - 11: Sixth International Symposium on Field-Flow Fractionation, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy. Tel: 39 532 291154; FAX: 39 532 240709.

SEPTEMBER 9 - 11: 110th AOAC International Annual Meeting & Expo, Hyatt, Orlando, Florida. Contact: AOAC International, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, USA. Tel: (703) 522-3032; FAX: (703) 522-5468.

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

SEPTEMBER 10 - 12: International Thermophiles Workshop: Keys to Molecular Evolution & the Origin of Life, Athens, Georgia. Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; FAX: (706) 542-2651.

SEPTEMBER 11 - 13: Corn Refiners Assn Scientific Conference, Bloomindale, Illinois. Contact: Corn Refiners Assn, 1701 Pennsylvania Ave, NW, Washington, DC 20036, USA. Tel: (202) 331-1634; FAX: (202) 331-2054.

SEPTEMBER 11 - 13: 2nd Workshop on Biosensors & Biol Techniques in Environmental Analysis, Lund, Sweden. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland.

SEPTEMBER 15 - 20: 21st International Symposium on Chromatography, Stuttgart, Germany. Contact: Dr. L. Kiessling, Geselleschaft Deutscher Chemiker, Postfach 900440, D-60444 Frankfurt/Main, Germany.

SEPTEMBER 16 - 18: 3rd International Conference on Applications of Magnetic Resonance in Food Science, Nantes, France. Contact: Laboratoire de RMN-RC, 2 rue de la Houssiniere, 44072 Nantes cedex 03, France. Tel: 33 40 74 98 06; FAX: 33 40 37 31 69.

SEPTEMBER 16 - 19: International Ion Chromatography Symposium 1996, University of Reading, Reading, UK. Contact: J. R. Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052, USA. Tel: (508) 359-8777; FAX: (508) 359-8778.

SEPTEMBER 17 - 20: 10th International Symposium on Cap[illary Electrophoresis, Prague, Czech Republic. Contact: Dr. B. Gas, Dept of Physical Chem, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic.

SEPTEMBER 19 - 21: 19th Annual Gulf Coast Chemistry Conference, Pensacola, Florida. Contact: J. Gurst, Chem Dept, University of West Florida, Pensacola, FL 32514, USA. Tel: (904) 474-2744; FAX: 904) 474-2621.

SEPTEMBER 20 - 24: 12th Asilomar Conference on Mass Spectrometry, Pacific Grove, California. Contact: ASMS, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4517; FAX: (505) 989-1073.

SEPTEMBER 28 - OCTOBER 5: Federation of Analytical Chem & Spectroscopy Societies (FACSS), Kansas City. Contact: J.A. Brown, FACSS, 198 Thomas Johnson Dr., Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 17 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

OCTOBER 27 - 31: American Assn of Pharmaceutical Scientists Annual Meeting & Expo, Seattle, Washington. Contact: AAPS, 1650 King St, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000;

OCTOBER 29 - 30: ASTM Symposium on Pesticide Formulations & Application Systems, New Orleans, Louisiana. Contact: G. R. Goss, Oil-Dri Corp, 777 Forest Edge Dr, Vrenon Hills, IL 60061, USA. Tel: (708) 634-3090;

OCTOBER 29 - 31: Cphl Pharmaceutical Ingredients Worldwide '96 Conference, Milan, Italy. Contact: Miller Freeman BV, Industrieweg 54, P. O. Box 200, 3600 AE Maarssen, The Netherlands. Tel: 31 3465 73777; FAX: 31 3465 73811.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 6 - 9: 24th Biennial International Conference on Application of Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan or B. Stippec, University of North Texas, Physics Department, Denton, TX 76203, USA. Tel: (817) 565-3252; FAX: (817) 565-2227.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Hyatt Regency Hotel, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

NOVEMBER 10 - 13: 4th North American Research Conference on Organic Coatings Science & Technology, Hilton Head, South Carolina. Contact: A. V. Patsis, SUNY, New Paltz, NY 12561, USA. Tel: (914) 255-0757; FAX: (914) 255-0978.

NOVEMBER 10 - 14: 10th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Kluczynski, Electrosynthesis Co, 72 Ward Road, Lancaster, NY 14086, USA. Tel: (716) 684-0513; FAX: (716) 684-0511.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

NOVEMBER 11 - 20: 2nd Latin-American Conference on Biomedical, Biopharmaceutical and Industrial Applications of Capillary Electrophoresis, Santiago, Chile. Dr. E. Guerrero, Servicio Medico Legal, Avenida de la Paz 1012, Santiago, Chile.

NOVEMBER 12 - 14: Plastics Fair, Charlotte, North Carolina. Contact: Becky Lerew, Plastics Fair, 7500 Old Oak Blvd, Cleveland, OH 44130, USA. Tel: (216) 826-2844; FAX: (216) 826-2801.

NOVEMBER 13 - 15: 13th Montreux Symposium on Liquid Chromatography-Mass Spectrometry (LC/MS; SFC/MS; CE/MS; MS/MS), Maison des Congres, Montreux, Switzerland. Contact: M. Frei-Hausler, Postfach 46, CH-4123 Allschwill 2, Switzerland. Tel: 41-61-4812789; FAX: 41-61-4820805.

NOVEMBER 18 - 20: 3rd International Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: Chem in Industry Conf, P. O. Box 1723, Dhahran 31311, Saudi Arabia. Tel: 966 3 867 4409; FAX: 966 3 876 2812.

NOVEMBER 17 - 22: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S., Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218; FAX: (302) 738-5275.

NOVEMBER 24 - 27: Industrial Research for the 21st Century, 1996 Biennial Meeting, Red Lion Resort, Santa Barbara, California. Contact: R. Ikeda, DuPont Central Research Dept., P. O. Box 80356, Wilmington, DE 19880-0356, USA. Tel: (302) 695-4382; FAX: (302) 695-8207; Email: ikeda@esvax.dnet.dupont.com.

DECEMBER 17 - 20: First International Symposium on Capillary Electrophoresis for Asia-Pacific, Hong Kong. Contact: Dr. S. F. Y. Li, Dept of Chemistry, National University of Singapore, Kent Ridge Crescent, Singapore 119260, Republic of Singapore.

1997

APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

APRIL 14 - 19: Genes and Gene Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California. Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California. Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994, USA. Tel: (517) 496-6491; FAX: (517) 496-6824.

MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY. Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd, Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois. Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York. Contact: T. Noce, Rust Envir & Infrastructure, 12 Metro Park Rd, Albany, NY 12205, USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas. Contact: J. H. Rigby, Chem Dept, Wayne State Univ., Detroit, MI 48202-3489, USA. Tel: (313) 577-3472.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.ord.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. FAX: (314) 341-6033.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218; FAX: (302) 738-5275.

1998

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA. Email: reglmtgs@acs.org.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Resw Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

1999

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 23: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2003

MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2005

MARCH 13 - 17: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 1: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

The **Journal of Liquid Chromatography & Related Technologies** will publish, at no charge, announcements of interest to scientists in every issue of the journal. To be listed in the Liquid Chromatography Calendar, we will need to know:

a) Name of the meeting or symposium,

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c) When and where it will be held, and

d) Whom to contact for additional details.

Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography & Related Technologies, P. O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

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Author(s)' names and full addresses
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Text Discussion
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F. D. Pierce, H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

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4. **Text Discussion**: Whenever possible, the text discussion should be divided into major sections such as

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METHODS
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6. **Acknowledgments**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

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Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

Book:

1. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York, 1979.

Chapter in a Book:

1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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J. LIQ. CHROM. & REL. TECHNOL., 19(12), (1996)

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JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

Volume 19, Number 12, 1996

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