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Identification and quantitation of drugs of abuse in urine using the generalized rank annihilation method of curve resolution

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Abstract

A rapid analytical method which is of practical use for the identification and quantitation of drugs of abuse in urine using HPLC with a diode-array detection is described. Because the method utilizes mathematical resolution of partially resolved peaks, greatly simplified sample preparation procedures and very short run times can be used. The generalized rank annihilation method (GRAM) is used to eliminate response due to unknown background peaks and separate partially resolved peaks. An optimized gradient elution program was found for which morphine, phenylpropanolamine, ephedrine, benzoylecgonine, lidocaine, cocaine, diphenhydramine, nortriptyline, norpropoxyphene, nordiazepam, codeine, D-amphetamine, meperidine, and amitriptyline elute from the HPLC column in less than 8.5 min. A commercially available system for HPLC analysis of drugs of abuse is currently available, however, the commercially available system takes 21 min to complete its analysis. Two modified sample pre-treatment methods were also developed to simplify sample treatment procedures substantially. In this paper, The GRAM technique is shown to be extremely powerful in identifying drugs of abuse from large overlapping peaks.

1. Introduction

Urinary drug analysis, whether for postmortem forensic purposes, pre-employment screening, or clinical purposes, is an expanding area of commercial interest for which rapid, reproducible analytical techniques are of great value [1,2]. In this paper, a rapid HPLC–diode-array (HPLC–DA) method for identification and quantitation of drugs of abuse in urine samples is reported. Diode-array detectors for HPLC can significantly improve the specificity of the tech-

nique. Two-dimensional data are generated for each component, allowing peak identification through retention time, wavelength ratioing, and spectral matching. Recently, Logan and co-workers reported a screening method for drugs of abuse in urine using cation-exchange solid-phase extraction and HPLC with diode-array detection [3]. In their publication, the experimental procedure was reported to be easily performed and gave excellent recoveries for all compounds investigated; however, the total time for the pre-treatment and analysis of each urine sample was lengthy. Modifications to the sample pre-treatment procedure and HPLC conditions

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were made to reduce the experimental time substantially. These reductions were made possible by use of a method for mathematical resolution and quantitation of overlapping peaks called the generalized rank annihilation method (GRAM) [4,5].

Because the samples are collected from patients' urine, there are many other endogenous and exogenous substances present, making it difficult to determine the constituents of interest (drugs of abuse) in these kinds of samples. In some cases, resolving the chromatographic peaks of the constituent of interest from interfering components is difficult via HPLC. In our studies, the GRAM is used to solve such curve resolution problems. Estimation of the spectra and concentration profiles of pure constituents is achieved by applying GRAM even though the chromatograms of the components in a complex mixture are overlapped severely.

Instruments that generate a matrix of data per sample (*e.g.* chromatography–spectroscopy, emission–excitation fluorescence) are called second-order instruments because the data sets produced by them can be represented by a second-order tensor [6]. The resulting data matrix is called a bilinear data matrix if the response at all wavelengths is linear with respect to concentration and if the response is equal to the sum of the response for each component. The resolution of spectra and concentration profiles of pure constituents from bilinear data by three-mode principal component analysis (*e.g.* rank annihilation or GRAM) has been recently developed. Appellof and Davidson [7] were the first to report an iterative method called rank annihilation (RA) for analyzing three-dimensional arrays of fluorescence excitation–emission matrices in which chromatographic retention time was used as the third dimension. Their algorithm was extended later by Russell and Gouterman [8–10] in the analysis of excitation–emission–lifetime arrays in which the time–decay profile of fluorescence following pulsed excitation was used as the third dimension. Good initial guesses for unknown spectra were required in this extension, and convergence depended heavily on the initial guesses. To date,

the RA method has been applied to LC–UV data [11], fluorescence excitation–emission spectroscopy [12,13] and thin layer chromatography–reflectance imaging spectrophotometry [14]. When there are analytes in an unknown sample that are not present in the standard or the concentrations of analytes are very low, the ordinary rank annihilation method often fails to work. For this reason, Sanchez and Kowalski developed generalized rank annihilation factor analysis (GRAFA) which was later called the generalized rank annihilation method (GRAM) [4,5]. In the application of GRAM to bilinear data, two sample matrices are required: One is the unknown sample and the other is the standard. By using GRAM, the pure component spectra and concentration profiles can be mathematically resolved uniquely and without iteration by using a single mixture of standards. Sanchez, Ramos and Kowalski applied new GRAM technique to LC–DA–UV data [15,16]. Recently, this new method was enhanced in our laboratory with similarity transformations [17,18].

2. Theory

When bilinear data are produced by an instrument, for example: liquid chromatography with a diode-array detector (LC–DA–UV), the response matrix N for a standard that contains one or more constituents can be expressed according to eq. 1:

$$N = \sum_{k=1}^K (\mathbf{x}_k c_{k,k} \mathbf{y}_k^T) = \mathbf{X} \mathbf{C}_N \mathbf{Y}^T \quad (1)$$

where \mathbf{X} and \mathbf{Y} are matrices whose columns \mathbf{x}_k and \mathbf{y}_k are vectors representing the pure constituent responses in the column space and row space of N , and \mathbf{C}_N is a diagonal matrix whose elements $c_{k,k}$ represent the concentration of the k th constituent in the standard sample. In the case of LC–DA–UV experiments, the \mathbf{y}_k vectors represent pure UV spectra and the \mathbf{x}_k vectors represent pure component elution profiles. The superscript T stands for the transpose of a vector or a matrix.

Usually our bilinear data are rectangular matrices. Each row of the data matrix represents one UV spectrum at a specific time. Each column represents an elution profile at a specific wavelength.

If \mathbf{X} and \mathbf{Y} are assumed to contain \mathbf{x}_k and \mathbf{y}_k for all constituents in the standard and unknown samples, then the response matrix for the unknown sample can be written according to eq. 2:

$$\mathbf{M} = \mathbf{X}\mathbf{C}_M\mathbf{Y}^T \quad (2)$$

If it is assumed that matrices \mathbf{X} and \mathbf{Y} are the same for both \mathbf{M} and \mathbf{N} , eqs. 1 and 2 are valid even when the standard and unknown samples do not have the same number of components, as long as the matrices \mathbf{X} and \mathbf{Y} include all the components present in both response matrices [15].

Sanchez and Kowalski developed the Generalized Rank Annihilation Method that permits simultaneous quantitation of analytes in a bilinear matrix using a single mixture of standards [4,5]. They showed that eqs. 1 and 2 can be solved by the generalized eigenproblem:

$$\mathbf{M}\mathbf{V} = \mathbf{N}\mathbf{V}\mathbf{\Lambda} \quad (3)$$

$$\mathbf{\Lambda} = \mathbf{C}_M\mathbf{C}_N^{-1} \quad (4)$$

where \mathbf{V} and $\mathbf{\Lambda}$ are matrices of eigenvectors and eigenvalues and the superscript -1 stands for the inverse of a matrix. For LC–UV–DA experiments, the retention times and peak shapes for the constituents present in \mathbf{M} and \mathbf{N} must be nearly identical in the two samples, otherwise, GRAM will not work.

The QZ algorithm developed by Molar and Stewart can be used to solve the generalized eigenproblem [19]. Because the QZ algorithm requires that \mathbf{M} and \mathbf{N} be square matrices, a necessary preliminary step is to transform the rectangular matrices \mathbf{M} and \mathbf{N} into square matrices [5]. The transformation must preserve the rank of \mathbf{M} and \mathbf{N} . To accomplish this transformation, it is necessary to determine two sets of orthonormal vectors \mathbf{p}_i and \mathbf{q}_i which are basis sets for the joint column and row spaces, respectively, of \mathbf{M} and \mathbf{N} . The \mathbf{p}_i are then used to form the columns of a matrix \mathbf{P} and \mathbf{q}_i are used to

form the columns of a matrix \mathbf{Q} . In the general case where one or more constituents present in the standard may not be present in the unknown and where one or more constituents present in the unknown may not be present in the standard, \mathbf{P} could be calculated from the concatenated matrix $(\mathbf{M}|\mathbf{N})$ and \mathbf{Q} from the concatenated matrix $((\mathbf{M})/(\mathbf{N}))$ by computing the singular value decomposition (SVD) of both concatenated matrices.

$$(\mathbf{M}|\mathbf{N}) = \mathbf{P}\mathbf{S}_1\mathbf{V}^T \quad (5)$$

$$\left(\frac{\mathbf{M}}{\mathbf{N}}\right) = \mathbf{U}\mathbf{S}_2\mathbf{Q}^T \quad (6)$$

Here \mathbf{P} , \mathbf{V} , \mathbf{U} and \mathbf{Q} are eigenvectors and \mathbf{S}_1 and \mathbf{S}_2 are matrices of singular values. Once \mathbf{P} and \mathbf{Q} are determined, the eigenproblem shown in eq. 3 can be solved via the QZ algorithm where \mathbf{M}_{PQ} and \mathbf{N}_{PQ} are substituted for \mathbf{M} and \mathbf{N} .

$$\mathbf{M}_{PQ} = \mathbf{P}^T\mathbf{M}\mathbf{Q} = (\mathbf{P}^T\mathbf{X})\mathbf{C}_M(\mathbf{Q}^T\mathbf{Y})^T \quad (7)$$

$$\mathbf{N}_{PQ} = \mathbf{P}^T\mathbf{N}\mathbf{Q} = (\mathbf{P}^T\mathbf{X})\mathbf{C}_N(\mathbf{Q}^T\mathbf{Y})^T \quad (8)$$

$$\mathbf{M}_{PQ}\mathbf{V} = \mathbf{N}_{PQ}\mathbf{V}\mathbf{\Lambda} \quad (9)$$

The eigenvectors and eigenvalues of eq. 9 are guaranteed to be real and greater than zero only when \mathbf{M}_{PQ} and \mathbf{N}_{PQ} are positive definite and nonsingular (e.g. square symmetric matrices having real eigenvalues with all l greater than zero) [20]. In fact both \mathbf{M}_{PQ} and \mathbf{N}_{PQ} are real, non symmetrical, square matrices, so in the GRAM technique, it can not be guaranteed that each eigenvalue and eigenvector will be real. When complex eigenvalues and eigenvectors are encountered, the results cannot be used to estimate pure component profiles. In our studies, we found that similarity transformations can be used to eliminate the imaginary part in the mathematically resolved spectra and profiles of pure constituents [17,18].

The mathematically resolved spectra and elution profiles \mathbf{X} and \mathbf{Y} whose constituents are present in both the standard and unknown can be calculated according to eqs. 10 and 11.

$$\mathbf{X} = \mathbf{P}\mathbf{N}_{PQ}\mathbf{V} \quad (10)$$

$$Y = Q((V)^+)^T \quad (11)$$

where the superscript $+$ stands for the pseudo inverse of a matrix. The columns of X and Y can then be compared with library response vectors for the analytes of interest for diagnostic purposes.

The retention times for the constituent of interest in the sample and standard matrix must be nearly identical in order for GRAM to work. When isocratic elution programs were used there was almost no change in retention time for each constituent between different runs; however, larger shifts in retention time occurred when gradient elution programs were used. An automatic peak matching method was developed to facilitate the use of GRAM in HPLC experiments even when gradient elution programs were used. The peak matching algorithm automatically selected a sub-matrix (window) that covered the expected elution window of the components of interest. For each component of interest, the shift in the retention time was estimated by measuring the difference between the retention time of the component in the unknown and standard samples. The unknown data matrix was then shifted to match the standard as closely as possible by deleting spectra at one end of the time window and adding spectra to the other end of the time window. After the adjustment, the position of the component of interest the standard usually matched fairly well ($ca. \pm 1.0$ s).

3. Experimental

3.1. Equipment

HPLC analysis was performed using a gradient pumping system (Spectra-Physics Model 8750; San Jose, CA, USA) operated at 1.5 ml/min, with a 20- μ l loop valve injector (Rheodyne Model 7125; Cotati, CA, USA). The HPLC column was a Lichrospher 5 μ m 60 RP-select B (Hewlett-Packard, Palo Alto, CA, USA; 250 \times 4 mm I.D.) column. LC-DA-UV experimental data were obtained by using an LKB Model 2140 photodiode-array detector (LKB, Bromma,

Sweden). Data were collected with the LKB Wavescan software package on a PC/XT type computer using a wavelength range of 190 nm to 370 nm. Data sets from the PC/XT computer were transferred for post-processing to an IBM RS/6000 340H workstation by floppy disk or by a high-speed network.

Computer software to perform the GRAM data analysis was written using MATLAB 4.0. (Mathworks, S. Natick, MA, USA) running on an IBM RS/6000 340H workstation. Copies of the research programs may be obtained from the authors by sending a pre-formatted MS-DOS or Macintosh 3.5 disk and a self-addressed return envelope. A copy of the MATLAB interpreter is required to run the software. Versions of MATLAB for Windows 3.1, Macintosh and workstation computers must be purchased from Mathworks. The total CPU time for processing a single sample was *ca.* 1 min. Manual intervention was required to operate the research programs; however, all of the manual operations are amenable to automation by appropriate programming.

3.2. Reagents

Acetonitrile (solution A; Fisher, Fair Lawn, NJ, USA) and potassium phosphate buffer solution (solution B, 0.05 M, pH 3.2) were used as the mobile phase. Two different sets of standards were used. The first set of standards contained morphine, phenylpropanolamine, ephedrine, benzoylecgonine, lidocaine, cocaine, diphenhydramine, nortriptyline, norpropoxyphene and nordiazepam (Alltech, State College, PA, USA). The second set of standards contained morphine, ephedrine, codeine, D-amphetamine, benzoylecgonine, meperidine, cocaine, amitriptyline, norpropoxyphene and nordiazepam (Alltech). The stock standard solutions contained 1.0 mg/ml of the standard in methanol. Working standard solutions contained all ten components (50 μ g/ml, 10 μ g/ml).

3.3. Procedures

The purpose of this work was to develop a rapid analytical method which is of practical use

for identification and quantitation of drugs of abuse in urine. GRAM was used to resolve overlapping peaks, which permitted the use of shorter elution times.

Three sample pre-treatment procedures were tried. The first sample pre-treatment method and HPLC conditions were reported by Logan *et al.* [3]. Urine samples were extracted in the following manner: Strong cation-exchange columns (SCX, Analytichem, Harbor City, CA, USA) were conditioned under vacuum on a Vac Elut manifold (Varian, Sunnyvale, CA, USA) with methanol (2 ml), water (1 ml), and 10 mM phosphoric acid (0.5 ml). Urine (2 ml) and 10 mM phosphoric acid (1 ml) were mixed thoroughly and applied to the column. The column was washed with 10 mM phosphoric acid (1 ml), 0.1 M acetic acid (0.5 ml), and methanol (1 ml). The column was then air-dried for approximately 30 s and ammoniacal methanol (3%, 2 ml) was passed through the column and collected. In order to obtain adequate sensitivity we found it was necessary to evaporate the eluent to dryness in a speed vac concentrator for *ca.* 80–90 min and reconstitute it with 50 μ l of the initial mobile phase.

Initially HPLC analysis was performed using Logan's gradient program. The initial mobile phase composition was 10% acetonitrile (A) and 0.05 M potassium phosphate buffer (pH 3.2) (B). Both A and B were adjusted to 50% over 15 min. The gradient was reset to 10% A and 90% B from 15 min to 20 min. The column was allowed to re-equilibrate for 5 min before a new run was started.

Because the GRAM technique allows us to mathematically resolve partially overlapping peaks, we tried to reduce the experimental time by adjusting the chromatography conditions. In one set of experiments we tried an isocratic elution program with different ratios of acetonitrile and phosphate buffer solution. The following ratios of acetonitrile and phosphate buffer solution were tested: 55:45, 45:55, 35:65, 25:75, and 15:85 (v/v). In a second set of experiments we tried the gradient programs shown in Table 1. The flow-rate was 1.5 ml/min for all isocratic and gradient elution programs.

We also tried two modifications to the sample pre-treatment procedure. In the first modification to the sample pre-treatment method a 5-ml urine sample was pipetted into a centrifuge tube that contained 15 ml of distilled water. The urinary proteins were precipitated by the addition of 1.5 ml ZnSO_4 (100 g/l) and 0.8 ml of 0.1 M NaOH. The resulting mixture was centrifuged for 10 min at 5200 g. The supernatant liquid was filtered through a 0.45- μ m membrane filter to remove any remaining particulate matter. The resulting sample was injected using a 20- μ l injection loop. A spiked urine sample was prepared by pipetting a standard morphine solution (0.25 ml, 1 μ g per milliliter of methanol) and drug-free urine (4.75 ml) into a centrifuge tube that contained 15 ml of distilled water. After precipitation of urinary proteins with ZnSO_4 and 0.1 M NaOH, the supernatant liquid was centrifuged and filtered giving a concentration of morphine in the filtrate of 11.2 μ g/ml.

In the second modification to the sample pre-

Table 1
Gradient elution programs tested in optimization experiments

Gradient	Initial composition	Final composition at time (min)	Hold time (min)	Reset composition at time (min)	Equilibration time (min)	Total run time (min)
A	10% A, 90% B	50% A, 50% B, 15	0	10% A, 90% B, 20	5	25
B	10% A, 90% B	50% A, 50% B, 10	0	10% A, 90% B, 15	5	20
C	10% A, 90% B	50% A, 50% B, 5	0	10% A, 90% B, 10	10	20
D	10% A, 90% B	50% A, 50% B, 6	0	10% A, 90% B, 12	8	20
E	10% A, 90% B	75% A, 25% B, 7	3	10% A, 90% B, 15	5	20

A = acetonitrile; B = potassium phosphate buffer (pH 3.2).

treatment method a 1-ml urine sample was pipetted into a centrifuge tube. The urinary proteins were precipitated by the addition of 0.5 ml trichloroacetic acid (1%). The resulting mixture was centrifuged for 10 min at 5200 g. The supernatant liquid was filtered through a 0.2- μ m non-sterile filter to remove any remaining particulate matter. The resulting sample was injected using a 20- μ l injection loop. Urine samples were prepared by splitting a drug-free urine sample into three parts. Each part was spiked with the mixture of ten drugs to give concentrations of each drug of 10 μ g/ml, 25 μ g/ml and 50 μ g/ml, respectively. One patient's urine sample that was positive for morphine and benzoylecgonine was split into two parts. One part was spiked with the mixture of ten drugs to give concentrations of each drug in the spiked urine sample of 20 μ g/ml, however, the concentration was obviously higher for the drugs the patient tested positive for.

4. Results and discussions

When the sample preparation procedures described by Logan *et al.* [3] were used, the first set of standards was used (100 μ g/ml of each). Fig. 1 shows the bilinear spectro-chromatogram of the mixture of ten pure drugs. Fig. 2 shows the chromatogram of this sample. Excellent quantitative results were obtained when Logan's sample preparation procedure was used, however, long experimental times were required.

In the experiments to optimize the HPLC conditions and sample preparation procedures, the second set of the standards was used. Chromatograms obtained with isocratic mobile phases of 55:45 and 15:85 (v/v) are shown in Figs. 3A and B respectively. When the mobile phase of 55:45 acetonitrile–phosphate buffer was used some of the constituents of interest were partially overlapped by the solvent front. GRAM analysis of peaks overlapped by the solvent front did not work well. We presumed this was due to the non-linear response in this region near the solvent front caused by rapid changes in the

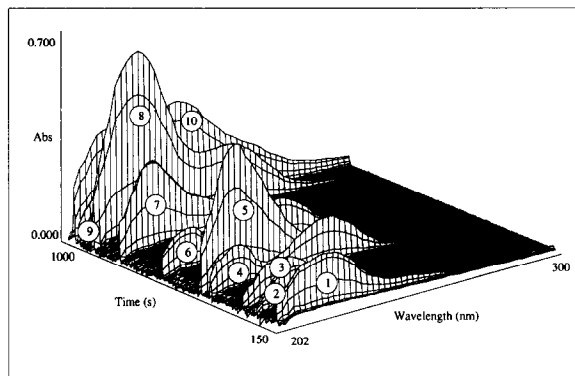


Fig. 1. Spectro-chromatogram of the first standard set. Peaks: 1 = morphine; 2 = phenylpropanolamine; 3 = ephedrine; 4 = benzoylecgonine; 5 = lidocaine; 6 = cocaine; 7 = diphenhydramine; 8 = nortriptyline; 9 = norpropoxyphene; 10 = nordiazepam. Initial mobile phase composition: 10% acetonitrile (A), 90% 0.05 M potassium phosphate buffer (pH 3.2) (B). Both A and B were adjusted to 50% over 15 min. The flow-rate was 1.5 ml/min.

refractive index of the eluent. When the mobile phase of 15:85 acetonitrile–phosphate buffer was used some of the constituents of interest did not elute at all. None of the isocratic conditions provided satisfactory results.

Gradient programs were then tested. Five different gradient programs were tried (see Table 1). We found the chromatogram using gradient program E was the best. Fig. 4 shows a sample chromatogram of the ten-component standard mixture using gradient program E. Under these

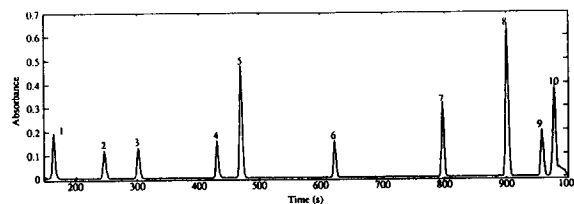


Fig. 2. Chromatogram of the first standard set. Peaks: 1 = morphine; 2 = phenylpropanolamine; 3 = ephedrine; 4 = benzoylecgonine; 5 = lidocaine; 6 = cocaine; 7 = diphenhydramine; 8 = nortriptyline; 9 = norpropoxyphene; 10 = nordiazepam. The HPLC conditions were identical to those used in Fig. 1.

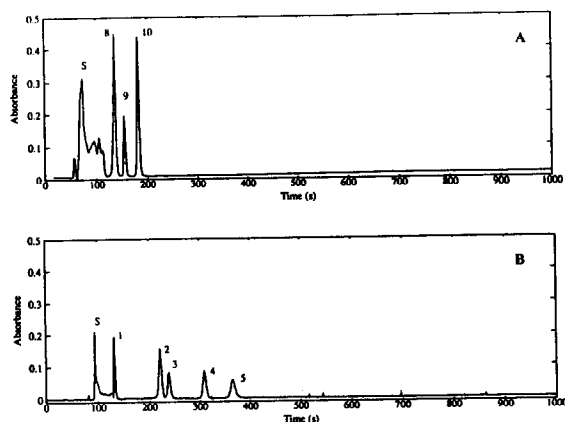


Fig. 3. Chromatograms of the second standard set using different isocratic programs. Peaks: s = solvent front; 1 = morphine; 2 = ephedrine; 3 = codeine; 4 = D-amphetamine; 5 = benzoylecgonine; 6 = meperidine; 7 = cocaine; 8 = amitriptyline; 9 = norpropoxyphene; 10 = nordiazepam. The ratio of acetonitrile and potassium phosphate buffer solution (v:v) was A: 55:45; B: 15:85. The flow-rate was 1.5 ml/min.

conditions, all components of interest elute from the HPLC column in less than 8.5 min and partial resolution of each drug is achieved.

Fig. 5 shows bilinear spectra for the morphine peak in the spiked urine and standard morphine samples. The concentration of morphine in the standard sample was 50 $\mu\text{g/ml}$. Fig. 6 shows the chromatograms of morphine in both the urine and standard samples at 208 nm. Morphine can be observed in the spectra of the urine sample

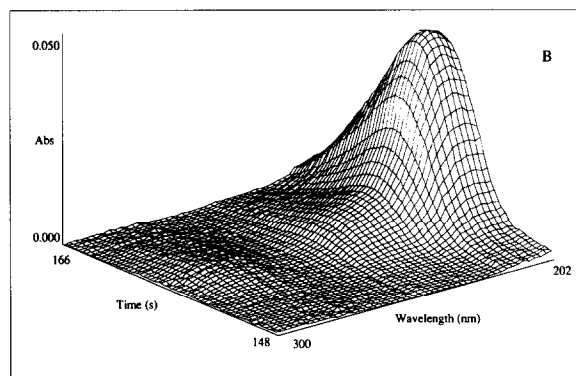
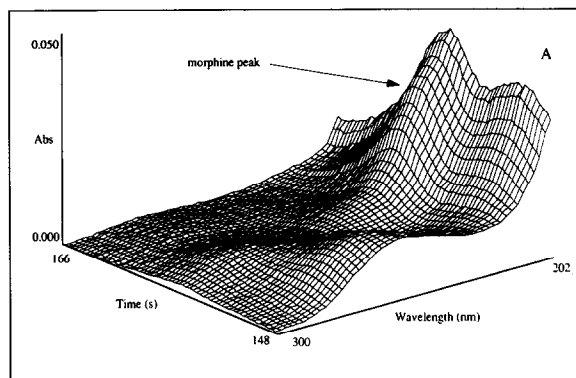


Fig. 5. Three dimensional bilinear absorption spectra for the spiked urine (A) and standard (B) samples in the analysis of morphine. The HPLC conditions were identical to those used in Fig. 4.

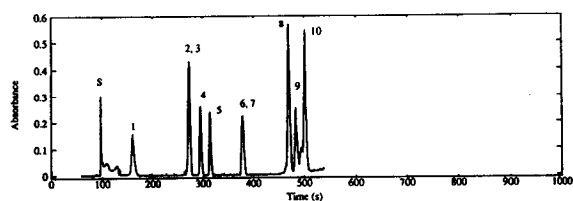


Fig. 4. Chromatogram of the second standard set using gradient program E shown in Table 1. Peaks: s = solvent front; 1 = morphine; 2 = ephedrine; 3 = codeine; 4 = D-amphetamine; 5 = benzoylecgonine; 6 = meperidine; 7 = cocaine; 8 = amitriptyline; 9 = norpropoxyphene; 10 = nordiazepam. The flow-rate was 1.5 ml/min.

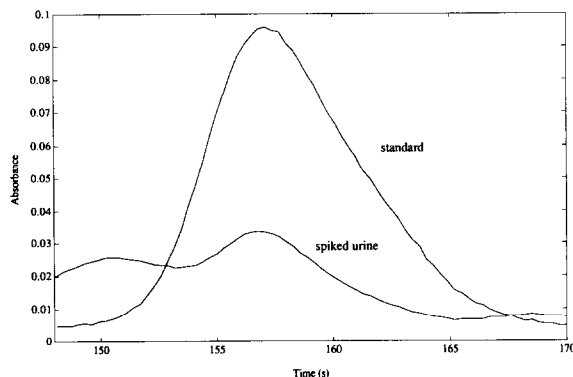


Fig. 6. Chromatograms from for morphine in the spiked urine and standard samples at 208 nm.

shown in Figs. 5A and 6, however, it was overlapped severely with other unknown interfering components that are present because the strong cation-exchange extraction step was omitted. The GRAM method worked very well in this example. The mathematically resolved chromatogram and UV spectrum for morphine in the spiked urine sample are shown in Fig. 7. The concentration of morphine in the spiked urine sample computed by GRAM was $11.1 \mu\text{g/ml}$ giving a nearly perfect recovery: 99.2%.

An example of the analysis of nordiazepam in urine using the modified sample pre-treatment method with trichloroacetic acid is presented. Fig. 8A shows the bilinear spectra of nordiazepam in a spiked urine sample ($10 \mu\text{g/ml}$), and Fig. 8B shows the bilinear spectra of the standard nordiazepam sample ($50 \mu\text{g/ml}$). Fig. 9 shows chromatograms of nordiazepam in both the spiked and standard samples at 226 nm.

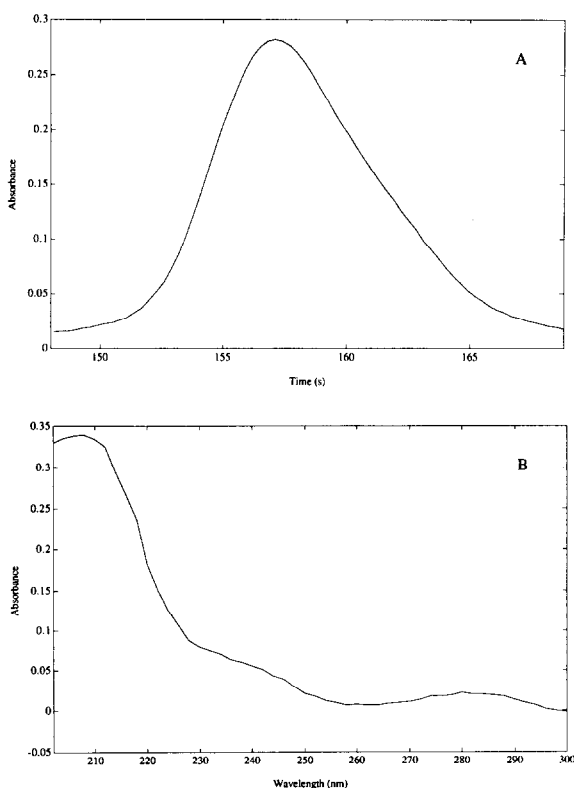


Fig. 7. Mathematically resolved concentration profile (A) and spectrum (B) for morphine in the spiked urine sample.

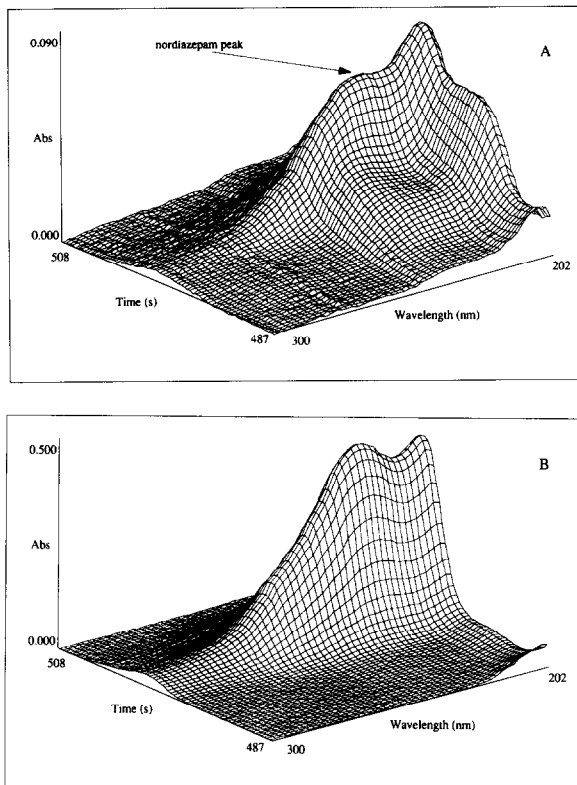


Fig. 8. Three dimensional bilinear absorption spectra for the spiked urine (A) and standard (B) samples in the analysis of nordiazepam. The HPLC conditions were identical to those used in Fig. 4.

Nordiazepam can be found in the urine sample (Figs. 8A and 9); however, like the previous example, it was overlapped severely with other

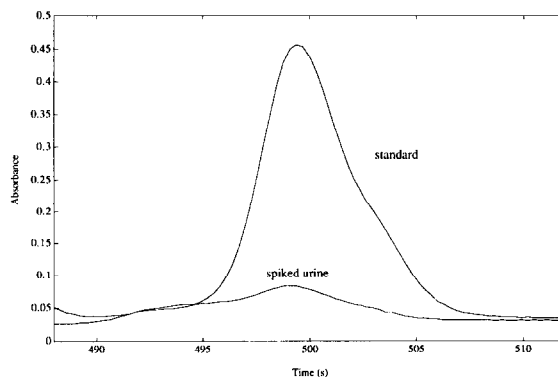


Fig. 9. Chromatograms for nordiazepam in the spiked urine and standard samples at 226 nm.

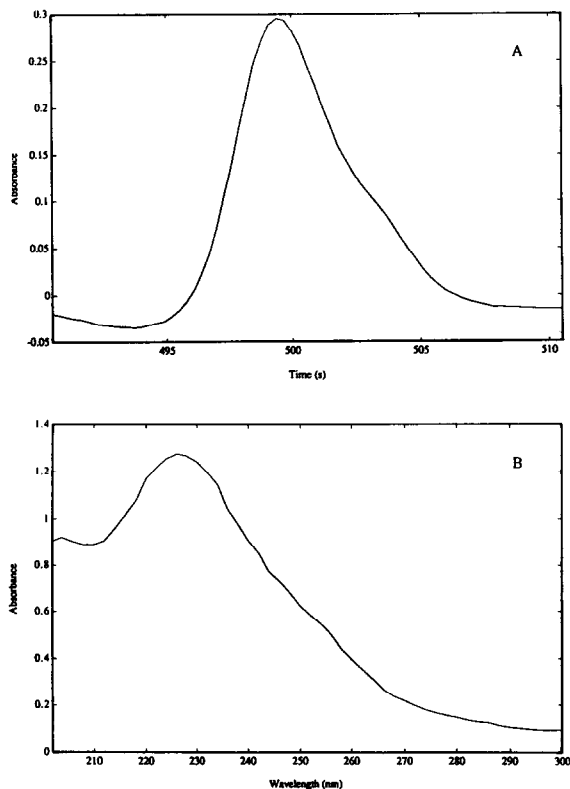


Fig. 10. Mathematically resolved concentration profile (A) and spectrum (B) for nordiazepam in the spiked urine sample.

interfering components that are present because the strong cation-exchange extraction step was omitted. The GRAM method also worked well in this example. The mathematically resolved chromatogram and UV spectrum for nordiazepam in the spiked sample are shown in Fig. 10. The concentration of nordiazepam in the spiked urine sample computed by GRAM was $8.66 \mu\text{g/ml}$ giving a satisfactory recovery of 86.6%.

We have applied our modified GRAM technique to the analysis of all ten drugs in all spiked and unspiked urine samples. The results for the concentrations of each drug in each sample are shown in Table 2. For the simultaneous determination of all ten drugs in the spiked drug-free urine samples the average bias ranged from a minimum of $-6.8 \mu\text{g/ml}$ to a maximum of $1.5 \mu\text{g/ml}$. The overall bias was $-1.3 \mu\text{g/ml}$, indicating that GRAM tends to underestimate the actual concentrations. Standard deviations for the determinations ranged from 1.0 to $10.4 \mu\text{g/ml}$. Table 3 shows the results of the recovery for each drug in the four spiked urine samples. In most cases, the recovery is close to 100%. In the analysis of morphine and benzoylecgonine that were tested to be positive in the patient's sample, the difference in the concentration of these

Table 2
Concentration of each drug in each urine sample

Sample	Concentration ^a ($\mu\text{g/ml}$)										Expected
	morp	ephe	code	amph	benz	mepe	coca	amit	norp	nord	
Spiked blank urine 1	9.1	8.3	9.2	11.0	10.3	9.4	10.3	9.3	9.2	8.7	10.0
Spiked blank urine 2	19.2	22.5	28.9	21.3	24.7	21.7	18.8	24.7	27.2	24.5	25.0
Spiked blank urine 3	60.1	51.9	39.9	50.7	54.5	43.7	36.6	48.7	50.0	45.9	50.0
Mean bias	1.2	-0.8	-2.3	-0.7	1.5	-3.4	-6.4	-0.8	0.5	-2.0	-1.3 (bias, overall)
S.D.	8.3	2.5	7.7	2.7	3.2	5.1	10.4	1.0	1.7	3.1	5.5 (S.D., overall)
Spiked patient urine	42.6 ^b	17.0	18.8	16.4 ^b	44.5	19.5	17.3	19.3	19.6	19.2	
Unspiked patient urine	26.7			25.0							
Difference	15.9			19.5							

^amorp = morphine, ephe = ephedrine, code = codeine, amph = D-amphetamine, benz = benzoylecgonine, mepe = meperidine, coca = cocaine, amit = amitriptyline, norp = norpropoxyphene, nord = nordiazepam

^bDrugs were tested to be positive in the patient's urine sample.

Table 3
Recovery of each drug in each urine sample

Sample	Recovery ^a (%)									
Sample	morp	ephe	code	amph	benz	mepe	coca	amit	norp	nord
Spiked blank urine 1	91.0	82.6	92.4	109.7	103.3	94.5	102.9	93.1	91.6	86.6
Spiked blank urine 2	77.0	90.1	115.4	85.2	98.8	86.9	75.4	98.6	108.8	97.8
Spiked blank urine 3	120.2	103.8	79.8	101.3	109.0	87.3	73.1	97.5	100.1	91.9
Spiked patient urine	79.5 ^b	85.1	94.0	81.8	97.6 ^b	97.7	86.3	96.3	97.8	96.0

^amorp = morphine, ephe = ephedrine, code = codeine, amph = D-amphetamine, benz = benzoylecgonine, mepe = meperidine, coca = cocaine, amit = amitriptyline, norp = norpropoxyphene, nord = nordiazepam.

^bDrugs were tested to be positive in the patient's urine sample.

drugs between the spiked patient's sample and the unspiked patient's sample is also in close agreement with the expected value.

5. Conclusions

The potential of GRAM in the identification and quantitation of drugs of abuse in urine has been demonstrated. In our experiments, an optimized gradient elution program was found which elutes all components of interest from the HPLC column in less than 8.5 min. Two modified sample pre-treatment methods were also found to simplify the sample treatment procedures substantially. With these modifications, the analysis time was reduced dramatically. The GRAM technique was shown to be extremely powerful in identifying drugs of abuse from large overlapping peaks.

In GRAM, it is necessary that the retention times of the constituent of interest are as close as possible in both the standard and unknown sample because slight shifts in the retention times may cause failure of the technique. At present the mathematics of GRAM is still the subject of research in our laboratory and other laboratories. Efforts are being made to derive and understand how retention time shifts and spectral shifts effect the precision and accuracy

of the quantitative results. Also studies are being performed to derive how random measurement errors, spectral similarity, chromatographic resolution, and relative concentration ratios effect the limit of detection, precision and accuracy of results.

In our experimental data, the widths of all chromatographic peaks are in the range of 15 to 20 s. We determined that when there is only a small shift (*ca.* less than ± 2.5 s) in the retention time for the constituent of interest between the standard and the unknown, the GRAM technique usually worked; in this study the retention times of the mathematically resolved profiles were identical to those of the standard. In some cases the GRAM technique still worked even when there was a shift in the retention time of $\pm (2.5\text{--}5.0$ s) for the constituent of interest between the standard and unknown; however, we think it will be necessary to use a preliminary treatment of the data matrices to get a better match between the retention times of the constituent in the standards and the unknowns when shifts larger than ± 2.5 s occur. Alternatively, we found that it is often possible to avoid mismatched retention times by using a thermostatted column and by running the mixed standard at frequent intervals during analysis of a batch of samples so that degradation in column performance over time does not adversely effect the data analysis step.

6. References

- [1] P.F. Dixon, *High Pressure Liquid Chromatography in Clinical Chemistry*, Academic Press, London, 1976.
- [2] D.M. Hercules, *Contemporary Topics in Analytical and Clinical Chemistry*, Vols. 1–2, Plenum Press, New York, 1977.
- [3] B.K. Logan, D.T. Stafford, I.R. Tebbett and C.M. Moore, *J. Anal. Toxicol.*, 14 (1990) 154.
- [4] E. Sanchez and B.R. Kowalski, *Anal. Chem.*, 58 (1986) 496.
- [5] B.E. Wilson, E. Sanchez and B.R. Kowalski, *J. Chemometrics.*, 3 (1989) 493.
- [6] T.B. Hirschfeld, *Anal. Chem.*, 52 (1980) 297A.
- [7] C.J. Appellof and E.R. Davidson, *Anal. Chem.*, 53 (1981) 2053.
- [8] M.D. Russell and M. Gouterman, *Spectrochim. Acta*, 44A (1988) 857.
- [9] M.D. Russell and M. Gouterman, *Spectrochim. Acta*, 44A (1988) 863.
- [10] M.D. Russell, M. Gouterman and J.A. van Zee, *Spectrochim. Acta*, 44A (1988) 873.
- [11] M. McCue and E.R. Malinowski, *J. Chromatogr. Sci.*, 21 (1983) 229.
- [12] C.-N. Ho, G.D. Christian and E.R. Davidson, *Anal. Chem.*, 52 (1980) 1071.
- [13] C.-N. Ho, G.D. Christian and E.R. Davidson, *Anal. Chem.*, 53 (1981) 92.
- [14] M.L. Gianelli, D.H. Burns, J.B. Callis, Christian, G.D. Christian and N.H. Anderson, *Anal. Chem.*, 55 (1983) 1858.
- [15] E. Sanchez, L.S. Ramos and B.R. Kowalski, *J. Chromatogr.*, 385 (1987) 151.
- [16] L.S. Ramos, E. Sanchez and B.R. Kowalski, *J. Chromatogr.*, 385 (1987) 165.
- [17] S. Li, J.C. Hamilton and P.J. Gemperline, *Anal. Chem.*, 64 (1992) 599.
- [18] S. Li and P.J. Gemperline, *J. Chemometrics*, 7 (1993) 77.
- [19] C.B. Molar and G.W. Stewart, *SIAM J. Numer. Anal.*, 10 (1973) 241.
- [20] G. Strang, *Linear Algebra and its Applications*, Academic Press, New York, 1976.