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Drug identification in biological matrices using capillary electrophoresis and chemometric software

K.A. Lilley*, T.E. Wheat

Thermo Capillary Electrophoresis, 8 Forge Parkway, Franklin, MA 02038, USA

Abstract

Drugs and their metabolites in biological specimens are analyzed by a variety of techniques. Capillary electrophoresis could provide another useful approach because of its unique selectivity and high resolving power. For routine use, however, rugged methods must be developed and combined with detection that confirms peak purity and identity in difficult sample matrices, such as, urine. In this study, capillary electrophoresis is used with diode array detection, and chemometric software is employed for spectral analysis. The software includes a series of chemometric tools. Principle Component Analysis and Iterative Target Transform Factor Analysis are used to inspect each electropherogram for spectral homogeneity of the peaks and to deconvolute comigrations. These algorithms are used to confirm the assay results. This approach is tested and demonstrated for the analysis of amphetamine and common interferences in human urine.

Keywords: Drugs

1. Introduction

The measurement of drugs and metabolites is a significant general problem in clinical, medical research and in pharmaceutical laboratories. Methods are developed for specific problems using the whole range of tools of the analytical chemist. Capillary electrophoresis (CE) is an attractive additional technique because of its unique selectivity and high resolving power.

As with any separation-based method, three problems must be solved. First, a method is developed to separate the analytes of interest from one another and from any interferences. Then, there must be a high level of confidence in the identification and in the assessment of purity of the peaks, especially in complex samples. Finally, the method must be shown to give accurate and precise quantitative results with real samples.

These problems are magnified with samples of biological origin, where the analytes are usually present at low levels in a complex matrix that may exhibit a variable composition.

To address these issues, an existing HPLC method development program, Unicam Diamond Optimization Software, was adapted for use with CE. This software provides several mathematical tools for developing and evaluating resolution, as well as for peak tracking by spectral analysis. This process has been reviewed [1–3], and its application to CE methods development has been described [3]. Beyond its utility in developing separations, the software provides tools for assessing peak purity and identity.

*Corresponding author.

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When CE separations are monitored with a diode array detector (DAD), spectral data can provide qualitative information. This approach has often been used to identify peaks by comparing the spectrum at the apex to a library of standards and to assess peak purity by comparing spectra across a peak. The chemometric algorithms in Diamond Software provide additional information. First, each peak is subjected to Singular Value Decomposition to not only assess purity but also to define the number of peak components. Then, Iterative Target Transformation Factor Analysis (ITTFA) reconstructs the spectra of the individual analytes. The reconstructions are then matched to the standard separation, designated to be the reference library. This procedure does not require prior knowledge of the sample [1]. It is, therefore, well-suited to the confirmation of peak purity and identity in complex samples. The process is illustrated in the present work for the analysis of amphetamine in urine.

In clinical laboratories testing for drugs of abuse, large numbers of urine specimens are screened using a battery of automated immunoassays for specific drugs, including amphetamine. Although immunoassays do demonstrate specificity, they will often show crossreactions with metabolites or other structurally related compounds [4–6]. In practice, many compounds can give a positive immunoassay for amphetamine, including over-the-counter dietary supplements, allergy and cold medications. Samples giving positive results must be confirmed by a second method, typically GC–MS. In this study, the high resolving power of CE is combined with chemometric data to provide an alternative discriminatory result.

2. Experimental

2.1. Capillary electrophoresis

The capillary electrophoresis system consisted of a Crystal 310 capillary electrophoresis system, a Crystal 240 diode array detector, Chromascan 3 software and Diamond Optimization Software (Thermo CE, Franklin, MA, USA).

2.2. Materials

The capillary was unmodified fused silica, 66.5 cm×75 μm, with the window at 41.5 cm (Polymicro Technologies, Phoenix, AZ, USA). Buffers were prepared from salts of the highest grade commercially available and 18 mΩ water (Barnstead Nanopure, Dubuque, IA, USA). Methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), amphetamine, phentermine, ephedrine, chlorpheniramine, 3,4-methylenedioxyamphetamine (MDA), phenylpropanolamine, pseudoephedrine and caffeine were from Sigma (St. Louis, MO, USA). Accubond Evidex solid-phase extraction cartridges were from J&W Scientific (Folsom, CA, USA). Isopropyl alcohol from Burdick and Jackson (Muskegon, MI, USA) and methanol and methylene chloride from Fisher (Fair Lawn, NJ, USA) were all of HPLC grade.

2.3. Samples

A working standard was prepared in 12.5 m*M* borate buffer, pH 9.3, with a concentration of 0.02 mg/ml for each component. Blank and amphetamine-spiked urine samples were prepared from a fresh, untreated urine from a drug-free subject. Urine samples were also obtained from a cold sufferer who was self-medicating with an over-the-counter formulation containing pseudoephedrine.

2.4. Sample preparation

Typical urine specimens were analyzed without sample pretreatment. Solid-phase extraction was performed on urines with amphetamine levels of less than 0.5 μg/ml and included a parallel blank. The procedure was performed according to the manufacturer's protocol. The prepared extract from 5 ml of urine was reconstituted in 50 μl of 12.5 m*M* sodium borate buffer, pH 9.3, for CE analysis.

2.5. Methods

Sodium borate buffer, at a concentration of 250 m*M* with respect to borate, was prepared and the pH was adjusted to 9.3 with sodium hydroxide. Run

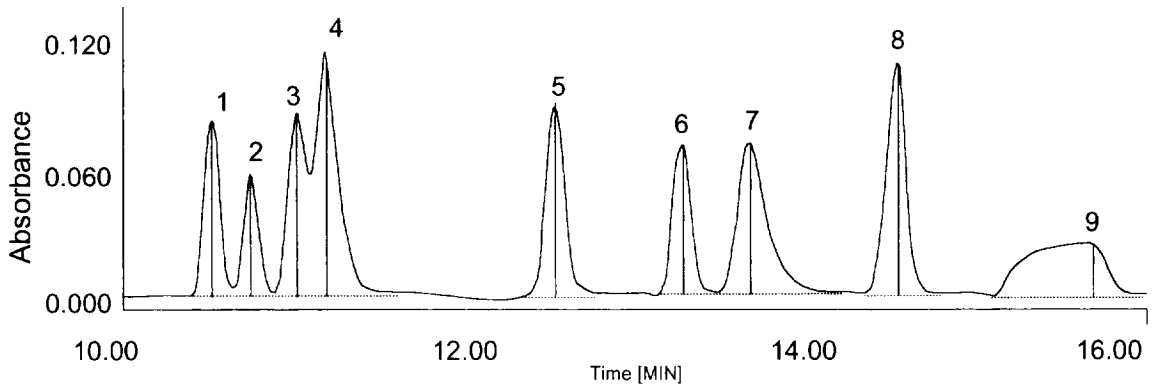

Fig. 1. Electropherogram of a standard mixture containing 0.02 mg/ml of each component. Conditions: 250 m*M* borate buffer, pH 9.3; capillary length, 66.5 cm (41.5 cm to window), 75 μm I.D. unmodified fused silica; 120 V/cm; temperature, ambient; detection at 195 nm; injection by positive pressure at 50 mbar/0.25 min; Peaks: 1=methamphetamine; 2=MDMA; 3=amphetamine; 4=phentermine and MDA comigration; 5=ephedrine; 6=chlorpheniramine; 7=phenylpropanolamine; 8=pseudoephedrine; 9=caffeine.

condition parameters, that varied ionic strength and pH, had been previously determined. This high molarity buffer at pH 9.3 was needed to achieve the separation. Injections were accomplished by positive pressure at 50 mBar for 0.25 min. A constant voltage of 8 kV was applied. The separations were performed at ambient temperature. Diode array detector spectra were collected at 0.8 point per second at 1.3 nm (full) resolution.

3. Results and discussion

The electropherogram of the standard mixture is shown in Fig. 1. Amphetamine and methamphetamine (peaks one and three) are the analytes of interest. The other compounds, with the exception of caffeine, can show significant crossreactivity in amphetamine screening immunoassays and are a potential source of false positives [4]. The chemo-

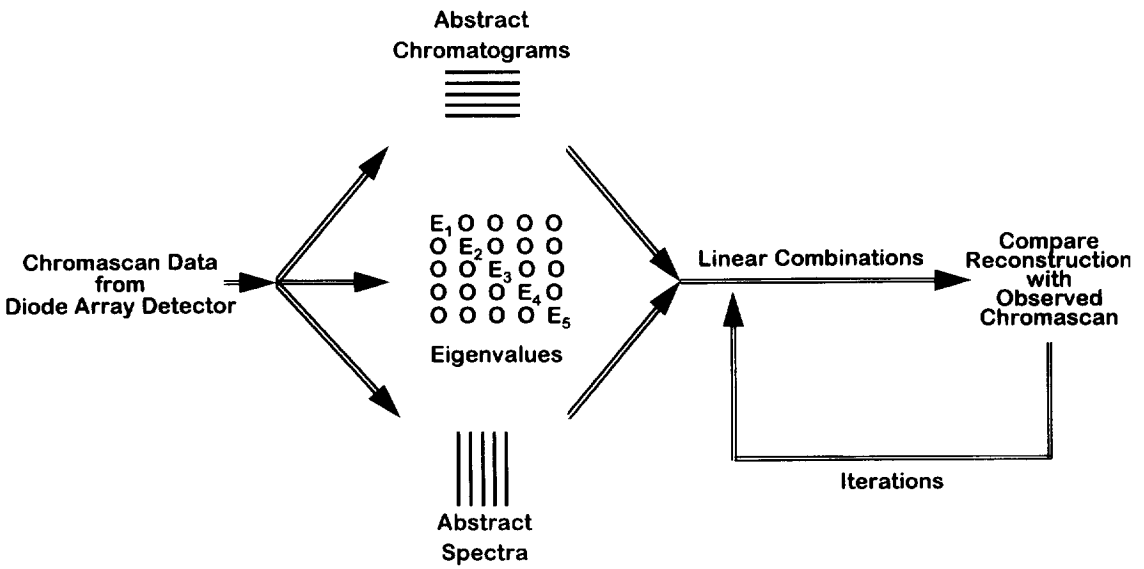

Fig. 2. Overview of Diamond Optimization Software chemometrics.

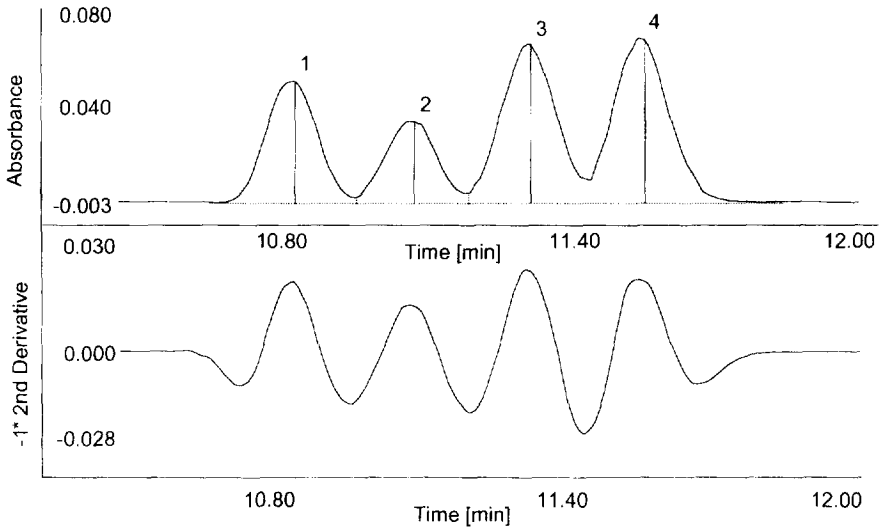

Fig. 3. Electropherogram at 195 nm. The lower portion of the figure shows the second derivative, which is plotted inverted, for ease of viewing. The second derivative's peaks and valleys are used by the software to set the targets within peaks for ITTFA and to define the boundaries of time segments in the electropherogram in the upper portion of the figure. Peaks: 1=methamphetamine; 2=MDMA; 3=amphetamine; 4=phentermine.

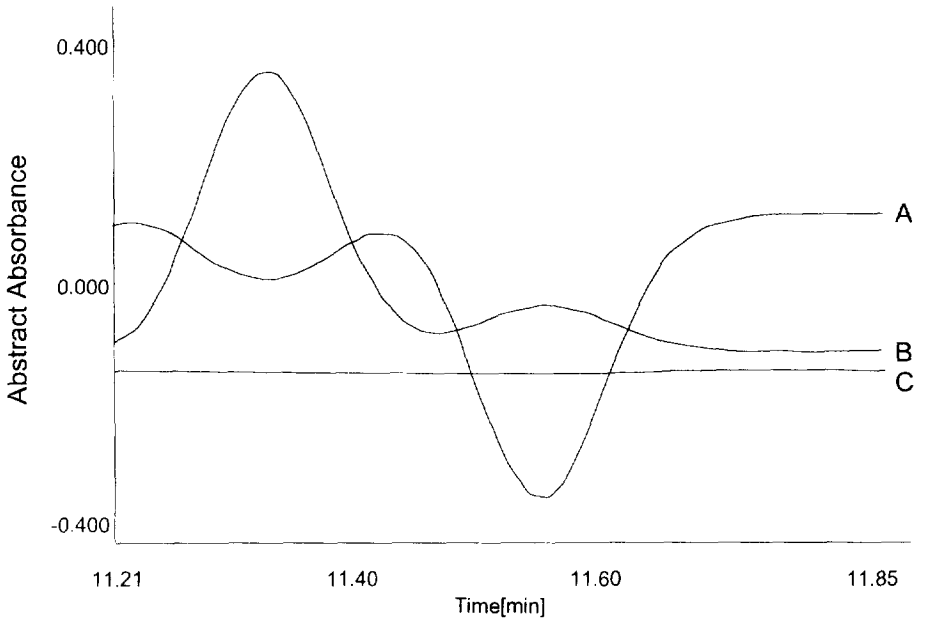

Fig. 4. Abstract chromatograms from a time segment that contains the amphetamine and MDA pair. The results of principle component analysis imply that three components are present with one component reflecting baseline noise and the other two indicating the presence of two real spectral elements. Peaks and valleys can help extrapolate the center of migration for each segment component. A,B=real components of time segment; C=baseline noise of time segment.

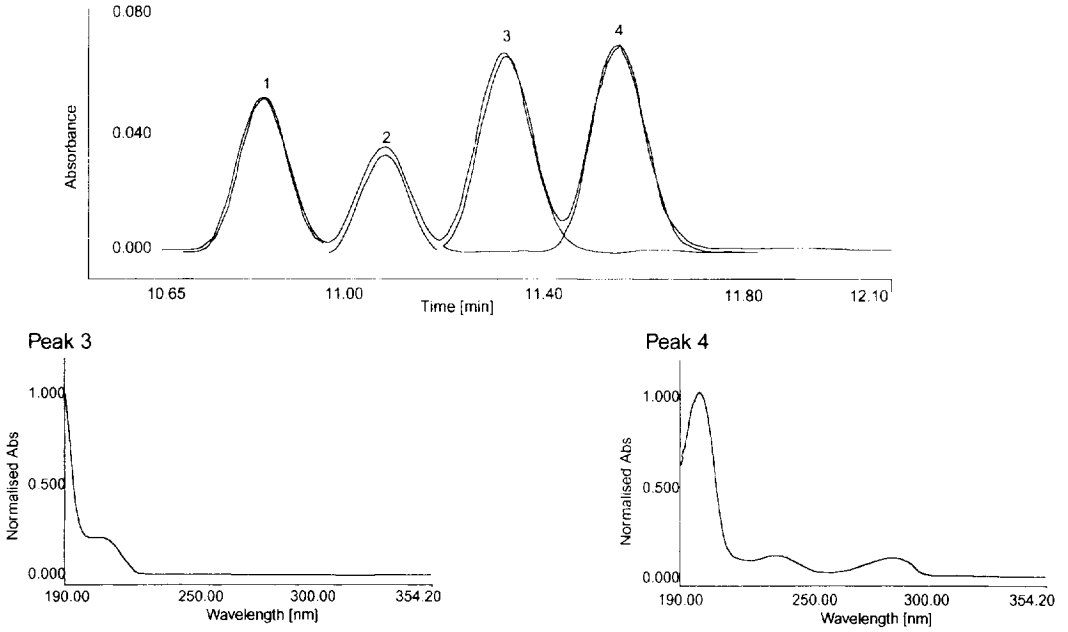

Fig. 5. The results of ITTFA, the reconstructed electropherograms are shown superimposed on the first four peaks. In this example, peaks three and four are not baseline-resolved, but can be easily differentiated by the software, based on spectral and migration time matches. Peaks: 1=methamphetamine; 2=MDMA; 3=amphetamine; 4=MDA.

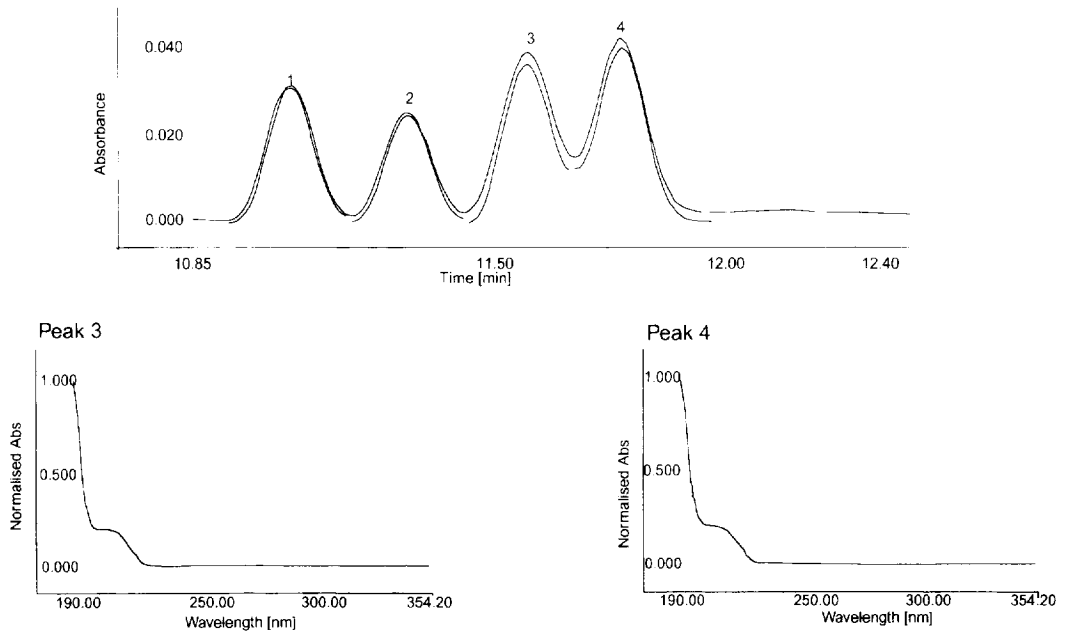

Fig. 6. Peaks three and four are not baseline-resolved. They also have matching spectra. If the time segment boundaries include both peaks, the reconstruction suggests that the peaks be linked. When the peaks are placed within individual time segments, the software uses a migration time match to differentiate them. The superimposed reconstructed electropherogram displays a close correlation to the original electropherogram peaks. Peaks: 1=methamphetamine; 2=MDMA; 3=amphetamine; 4=phentermine.

metric tools in Diamond Optimization Software that provide spectral deconvolution using principal component analysis are used to create a reference set for the analysis of unknown samples. This process is shown schematically in Fig. 2. The steps are illustrated in detail for the peaks migrating near 10 min.

First, the software extracts a maxplot electropherogram and calculates its second derivative (Fig. 3). Based on the second derivative, the software automatically divides the diode array data file into time segments containing peaks. Each second derivative apex is defined as an initial target for ITTFA, and the pair of valleys on either side mark the edges of the time segments for singular value decomposition. Each time segment is, mathematically, a three-dimensional matrix. Singular value decomposition extracts two two-dimensional matrices, abstract chromatograms and spectra, that are related by a diagonal matrix of eigenvalues. The number of eigenvalues is the number of components within that time segment. There is one eigenvalue for each analyte migrating in the time segment and one additional eigenvalue for baseline noise. If the value of each eigenvalue is known, the abstract spectra and chromatograms can be recombined to generate the original three-dimensional matrix. Since unknown eigenvalues cannot be calculated for a three-dimensional matrix, the software assigns an infinitely thin slice through the matrix as an initial target. The eigenvalues derived for this two-dimensional matrix are used across the time segment to reconstruct the individual components that contribute to the observed data. These estimated eigenvalues are iteratively refined by comparing successive trials to the original three-dimensional electropherogram. The software allows interactive approval of the reconstruction and the targets can be manually adjusted, based on the number of eigenvalues and the abstract chromatograms. This process is illustrated for the time segment with the amphetamine–MDA pair from 11.2 to 11.8 min.

This segment has three eigenvalues and three corresponding abstract chromatograms. One of these is for the baseline noise and the others are real analytes. In Fig. 4, the shape of the abstract chromatograms, especially the matching peaks and val-

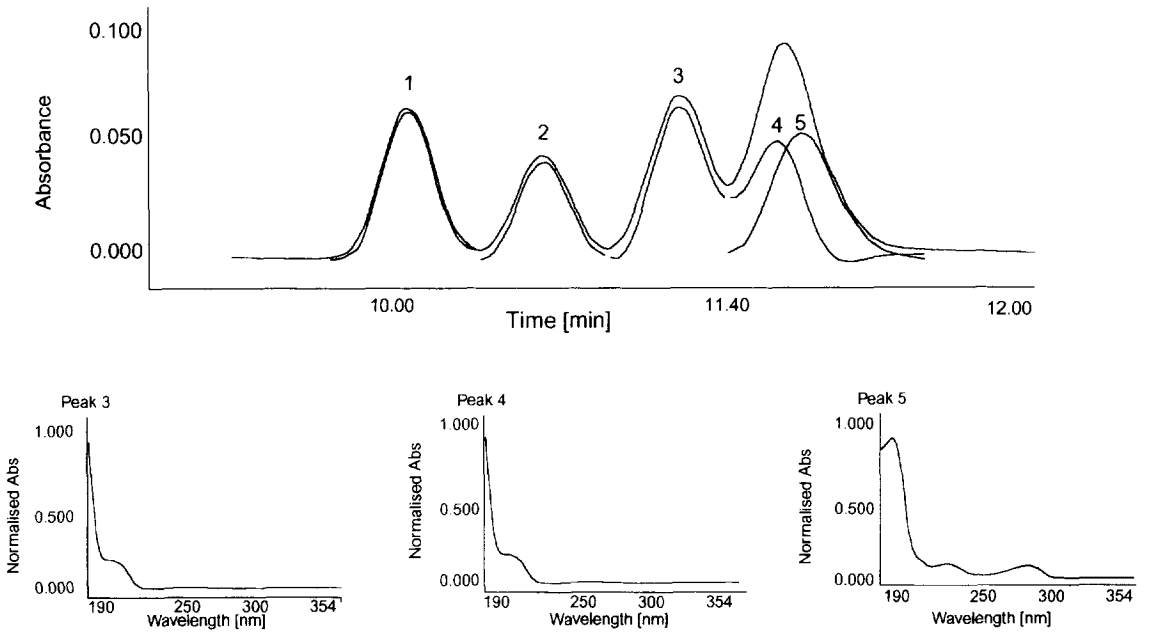

Fig. 7. The migration times for amphetamine, phentermine and MDA are nearly identical. With obvious valleys marking the boundaries of the two time segments that enclose these three peaks, three real components are suggested to be present. The superimposed reconstruction shows the comigrating peaks that were deconvoluted successfully because of spectral differences. Peaks: 1=methamphetamine; 2=MDMA; 3=amphetamine; 4=phentermine; 5=MDA.

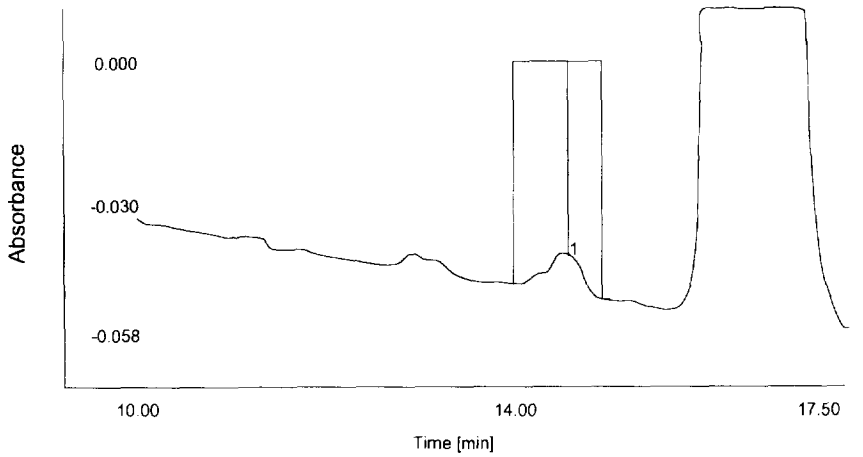

Fig. 8. Blank urine injection. No sample preparation. Conditions as in Fig. 2. No peaks corresponded to the reference set.

leys near 11.35 and 11.55 min indicate the likely center of migration for each component. These results form the basis for reconstruction by ITTFA. Each reconstructed peak is then stored with its spectrum, migration time and amount, as integral volume under the three-dimensional peak. The extracted pure spectra for amphetamine and MDA are shown in Fig. 5. The reconstructed standards are stored as a reference set. When unknown samples are deconvoluted in the same way, the reconstructions can be automatically matched to the reference set, based on migration time and spectrum.

The success of this deconvolution requires that there be both some electrophoretic resolution and

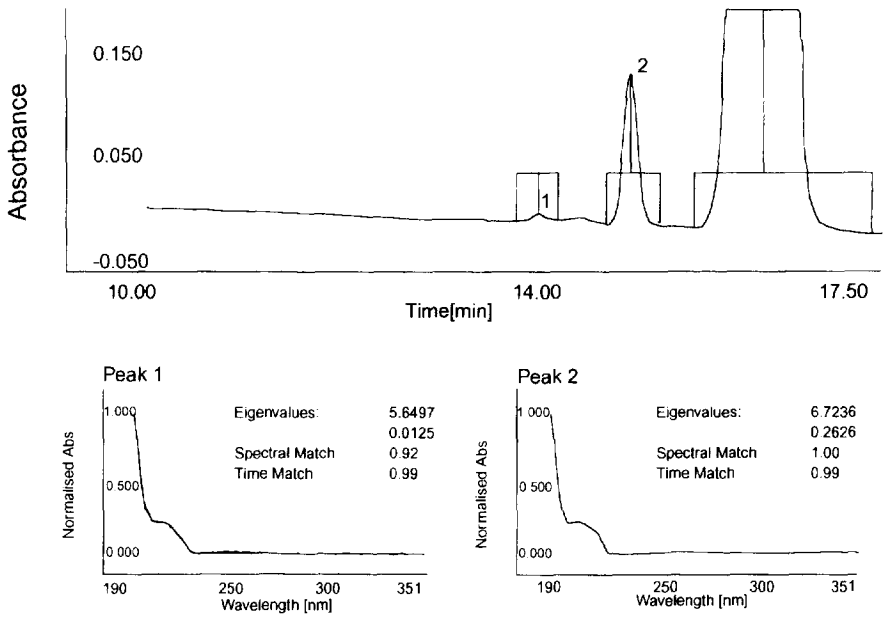

Fig. 9. Injection of urine from a person with a cold. No sample preparation. Conditions as in Fig. 2. Peaks 1 and 2 demonstrate near perfect spectral and time matches to the reference set components, phenylpropanolamine and pseudoephedrine. Peaks: 1=phenylpropanolamine; 2=pseudoephedrine.

some spectral difference between the comigrating species. The limits of this process can be illustrated for amphetamine and phentermine (Fig. 6). For these two compounds, the spectra are essentially identical. Therefore, the deconvolution treats them as a single component on its first pass. However, the time segment can be divided at the obvious valley between the peaks, to give a good reconstruction. These analytes would be distinguished from each other by migration time.

In the complete mix, amphetamine, phentermine and MDA form a closely spaced set. In Fig. 7, with the time segments divided at obvious valleys and one target assigned for each abstract chromatogram in the time segment, the reconstruction identifies all three components. The phentermine/MDA comigration is not at all apparent without this deconvolution. Thus, in a real sample giving a positive amphetamine immunoassay, an electrophoretic peak near 11.5 min would be identified as MDA primarily from its spectrum. Phentermine and amphetamine would be distinguished by their distinct migration times. This approach was tested on actual urine samples.

A urine sample typical of those that give a negative result in amphetamine immunoassays was analyzed (Fig. 8). It was optically clear and within the normal physiological pH range. It was not filtered, extracted or treated with any sample preparation. None of the trace peaks or baseline disturbances in this urine blank correspond to the reference compounds in either time or spectral properties. A second urine sample was obtained from a cold sufferer who was self-medicating with the recommended dose of an over-the-counter relief preparation containing pseudoephedrine. Such a sample would give a tentative positive result in a routine amphetamine screen and would require reanalysis with a procedure that demonstrated specificity. Once again, this sample was analyzed without sample preparation (Fig. 9). Two significant peaks were

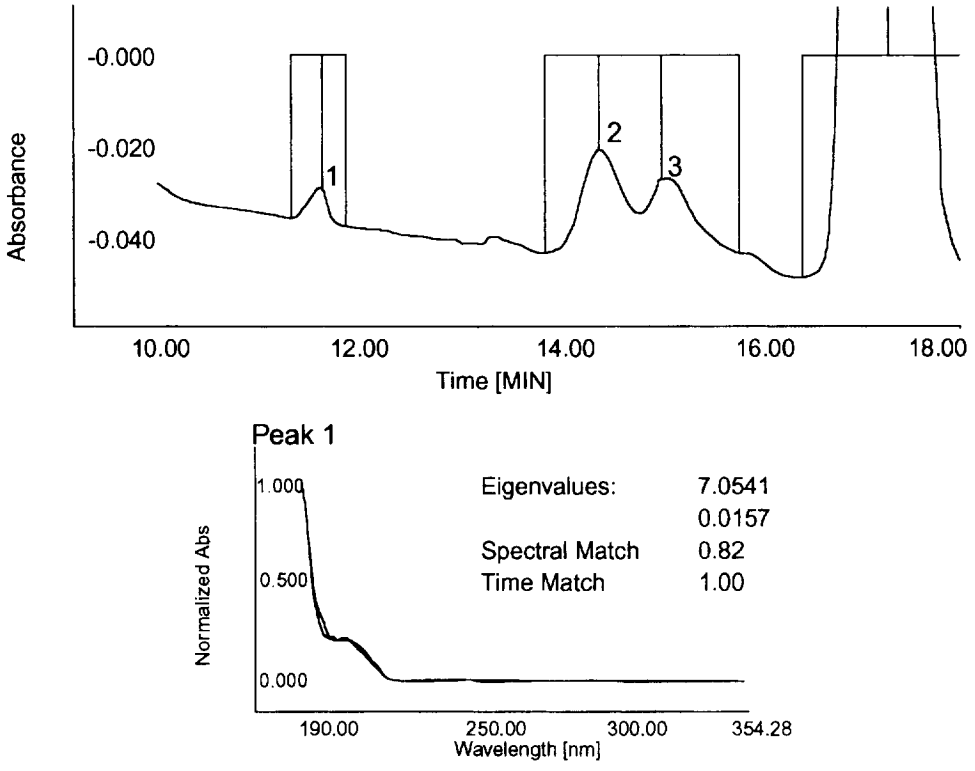

Fig. 10. Injection of urine spiked with 5 μg/ml of amphetamine. No sample preparation. Conditions as in Fig. 2. The software identifies peak 1 as amphetamine, based on migration time and spectral matching. Peak 1 and amphetamine spectra are shown superimposed with the software determining a spectral match coefficient of 0.82. Peaks: 1=amphetamine; 2=no match to reference set; 3=no match to reference set.

observed. Following deconvolution with Diamond Software, both peaks were readily identified. Both are pure since they have only two eigenvalues, one for the baseline noise and one for the analyte. They give good spectral and near perfect time matches to the reference compounds, pseudoephedrine and phenylpropanolamine. Phenylpropanolamine is present as a metabolite of pseudoephedrine. There is no evidence of amphetamine in this sample.

In contrast, a urine spiked with amphetamine at 5 μg/ml shows a clearly identified, homogenous peak for the drug, as shown in Fig. 10. This result was obtained on urine without sample preparation and at a sensitivity comparable to amphetamine immunoassays. The sensitivity and certainty should be improved with some sample preparation. When a spiked urine is carried through the same solid phase extraction that would be used to prepare the sample for GC–MS, the very simple electropherogram noted in Fig. 11, is observed. The first peak is unequivocally identified as amphetamine. The concentration of analyte in this urine is only 0.4 μg/ml. A satisfactory specific analysis is, therefore, possible at five times the sensitivity of typical immunoassays [6].

4. Conclusions

These results illustrate the feasibility of a novel approach to the general problem of analyzing small, bioactive molecules in biological samples. The intrinsically high resolution of HPCE is combined with chemometric analysis of diode array spectral data to

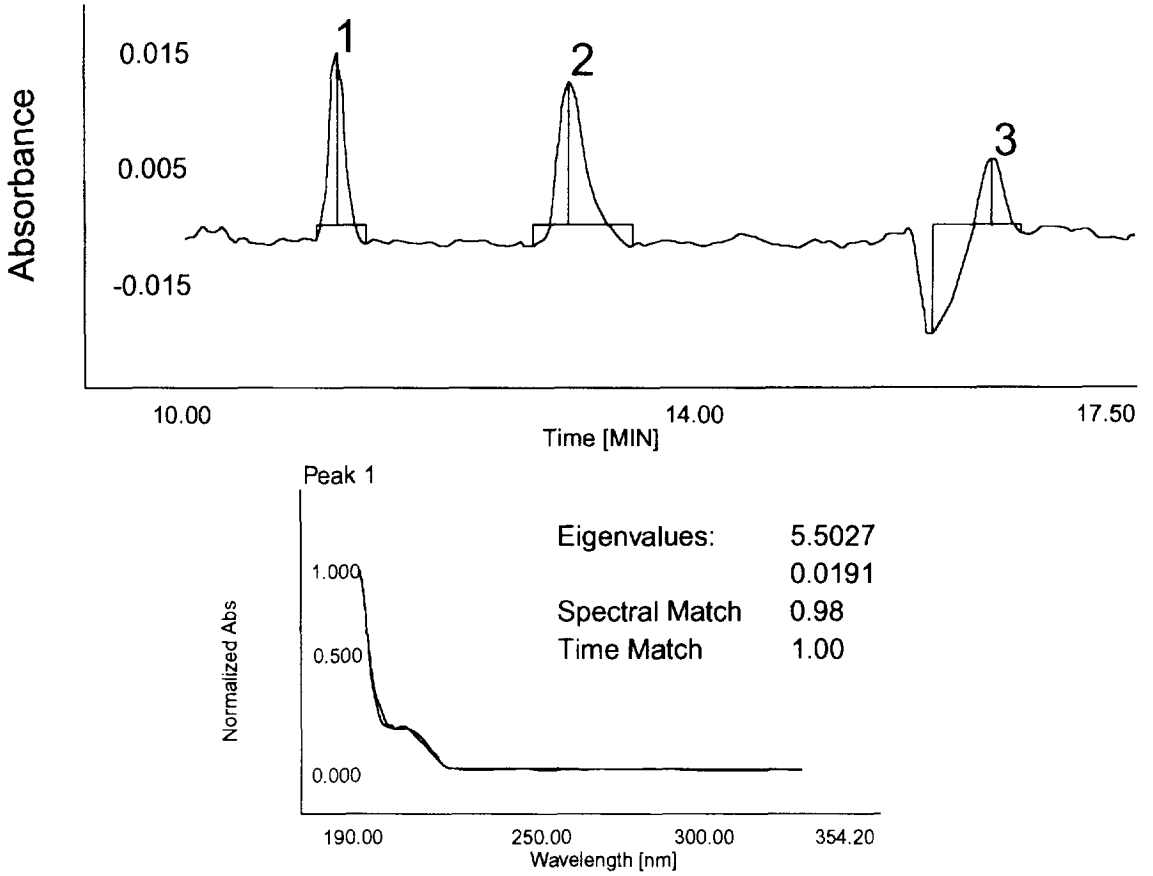

Fig. 11. Injection of urine spiked with 0.4 μg/ml of amphetamine. Solid phase extraction performed according to the cartridge manufacturer's instructions. Sample was reconstituted in 50 μl of 12.5 m*M* borate buffer, pH 9.3. Conditions as in Fig. 2. Peak one exhibits near perfect spectral and time matches to amphetamine. Peaks: 1=amphetamine; 2=no match to reference set; 3=no match to reference set.

confirm peak purity and identity. In this particular example, the application of this method to urine samples that are positive for amphetamines in immunoassays could have reduced the need for most of the confirmatory thin layer or GC–MS assays that would have been routinely applied to exclude false positives. Such false positives may include up to 80–90% of the urines initially found to be amphetamine positive in screens during the cold season. Only those samples with electrophoretic evidence of the drug would need to be carried to the confirmatory test. Since the extraction step was also eliminated, the savings in time and materials would be substantial. This general approach should be suitable with many other assays of drugs and metabolites in a wide range of clinical, pharmaceutical, and biomedical research applications.

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