Drug of Abuse Confirmation in Human Urine Using Stepwise Solid-Phase Extraction and Micellar Electrokinetic Capillary Chromatography

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This paper demonstrates that most common drugs of abuse can be adsorbed simultaneously onto a mixed-mode bonded-phase matrix and eluted sequentially in two to three steps for subsequent analysis by micellar electrokinetic capillary chromatography (MECC). Having on-column multiwavelength UV absorption detection, this is shown to be an attractive approach for confirmation testing of barbiturates, hypnotics, amphetamines, opioids, benzodiazepines, and metabolites of cocaine in a single aliquot of human urine. For these compounds, no hydrolysis of the urine specimen or sample derivatization is required. Under the examined conditions using 5 mL of urine, excellent recoveries (80–90% level) and detection limits (about 100 ng/mL) are obtained. For patient urines which tested positively for different classes of drugs using immunological screening methods, a two-step extraction scheme is shown to provide extracts suitable for rapid MECC confirmation of the drugs of abuse.

INTRODUCTION

Urine analysis has become an accepted method of investigating intoxications, identifying individuals using drugs of abuse, and controlling drug addicts following withdrawal therapy. Typically, urine samples are screened for drugs using immunoassays. Due to the lack of specificity of these techniques, positive results should be confirmed in order to eliminate any false-positive answer that may have resulted from the initial screening process. For that purpose drugs of abuse are extracted from the urine matrix (e.g. employing bonded-phase technology) prior to analysis with a highly specific method, including high-performance thin-layer chromatography (HPTLC), gas–liquid chromatography, high-performance liquid chromatography (HPLC), and gas chromatography/mass spectrometry (GC/MS).[1]

Recently, micellar electrokinetic capillary chromatography (MECC, an interface between electrophoresis and chromatography) was found to be an attractive approach for the analysis of drugs in body fluids, pharmaceutical preparations, and illicit seizure samples.[2-14] Using bonded-phase extraction procedures for specific classes of drugs followed by MECC with polychrome UV absorption detection was shown to be an effective method for the analysis of urinary barbiturates,[11] other illicit drugs and/or their metabolites, including opioids, benzoylecgonine (metabolite of cocaine), amphetamines, and methaqualone,[12] as well as cannabinoids.[13] Using this approach, the compounds have to be extracted, but not derivatized. Furthermore, with the exception of cannabinoids, no hydrolysis of the urine specimens is required, this providing the opportunity of monitoring relevant metabolites (including the heroin metabolite 6-acetylmorphine) which otherwise would be decomposed.[12] The characterization of each compound occurs via retention behavior and absorption spectrum. This work indicated that not all drugs can be resolved and analyzed in one run. For the analysis of many classes of drugs in a single urine specimen, this means that extracts have to be either analyzed sequentially in different buffers or that multiple, specific extraction schemes have to be employed. Alternatively, the search for a buffer configuration capable of resolving all important drugs could be considered.

Recently, disposable solid-phase extraction columns for the chromatographic analysis of acidic, neutral, and basic drugs from a single aliquot or urine or plasma have been developed.[15-18] This technique uses mixed-mode adsorbens exhibiting hydrophobic and ionic interactions. The objectives of the work described in this paper were (i) to investigate stepwise (digital) elution of urinary drugs after their adsorption onto a copolymeric bonded-phase matrix and subsequent analysis of each eluted fraction using MECC and (ii) to elucidate the pros and cons of this approach in comparison to the application of different buffers and extraction schemes.

MATERIALS AND METHODS

Chemicals, Origin of Samples, and Drug Screening. All chemicals used were of analytical or research grade. The drugs employed as reference compounds were of European Pharmacopeia quality. Urine samples were collected in our routine drug assay laboratory where they were received for drug screening. Our own urine was employed as blank matrix. The samples were screened for the presence of barbiturates, benzodiazepines, opiates, cocaine metabolites, methaqualone as well as amphetamines by automated enzyme immunoassay techniques (EMIT-dau, Syva, Palo Alto, CA) on a Cobas Fara centrifugal analyzer (F. Hoffmann-La Roche, Diagnostica, Basel, Switzerland) and stored at 4 °C until further analysis. The EMIT-dau tests contain calibrators with a cutoff level of 300 ng/mL each. Samples which

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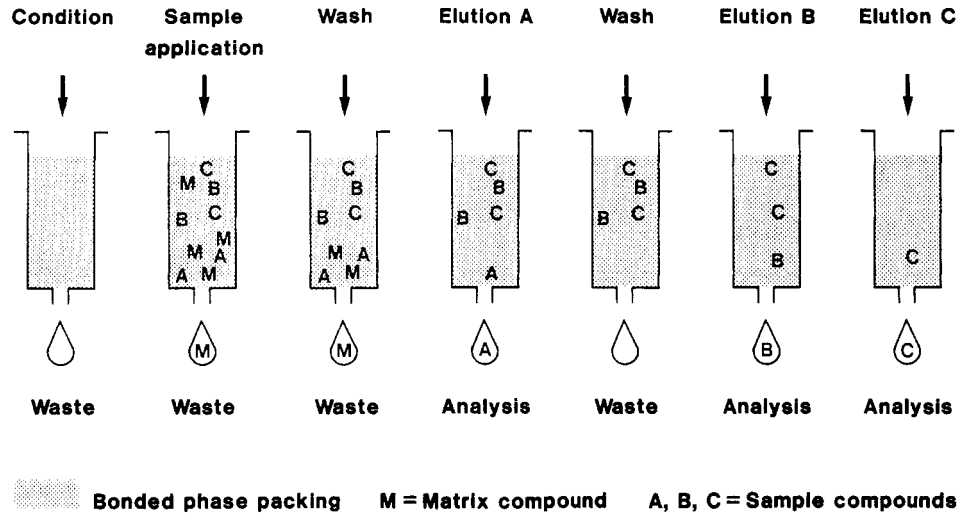

Figure 1. Schematic representation of a three-step bonded-phase extraction process from a single aliquot of urine.

gave an equal or higher response than the calibrators were interpreted as positive.

Electrophoretic Instrumentation and Running Conditions. The instrument with multiwavelength detection employed in this work was described previously.[11-13] Briefly, it featured a 75-μm i.d. fused-silica capillary of about 90-cm length (Product TSP/075/375, Polymicro Technologies, Phoenix, AZ) together with a fast scanning multiwavelength detector model UVIS 206 PHD with on-column capillary detector cell No. 9550-0155 (both from Linear Instruments, Reno, NV) toward the capillary end. The effective separation distance was 70 cm. A constant voltage of 20 kV was applied. The cathode was on the detector side. Sample application occurred manually via gravity through lifting the anodic capillary end, dipped into the sample vial, some 34 cm for a specified time interval (typically 5 s). Multiwavelength data were read, evaluated, and stored by employing a Mandax AT 286 computer system and running the 206 detector software package version 2.0 (Linear Instruments, Reno, NV) with windows 286 version 2.1 (Microsoft, Redmont, WA). Conditioning for each experiment occurred by rinsing the capillary with 0.1 M NaOH for 3 min and with buffer for 5 min. Throughout this work the 206 detector was employed in the high-speed polychrome mode by scanning from 195 to 320 nm at 5-nm intervals (26 wavelengths). Unless otherwise stated, a buffer composed of 75 mM sodium dodecyl sulfate (SDS), 6 mM $Na_2B_4O_7$, and 10 mM Na_2HPO_4 (pH about 9.1) was used.

Sample Pretreatment. Standard solutions of drugs of abuse were prepared in methanol at concentrations of 2000 μg/mL. Spiking of blank and patient samples occurred through addition of known aliquots of these standard solutions to the urine prior to extraction. Drugs were extracted using Bond Elut Certify cartridges No. 1211-3050 (sorbent amount, 130 mg; reservoir volume, 10 mL) and the Vac Elut setup (both from Analytichem International, Harbor City, CA).

Stepwise Extraction. The sequence of events employed for a three-step digital extraction scheme of substances A, B, and C is illustrated in Figure 1. The cartridges were conditioned immediately prior to use by passing sequentially 2 mL of methanol and an equal volume of 0.1 M phosphate buffer (pH 6) through the columns. The vacuum was turned off to prevent column drying. The columns were loaded by slowly (about 2 min) drawing of a mixture of 5 mL of urine and 2 mL of 0.1 M phosphate buffer (adjusted to pH 6). The columns were then rinsed sequentially with 1 mL of 0.1 M phosphate buffer/methanol (80:20), with 1 mL of 1 M acetic acid and with 1 mL of hexane (vacuum was held shortly at 5 mmHg). The first elution of drugs (sample A in Figure 1) occurred with 4 mL of methylene chloride into a clean test tube. The cartridge was then rinsed with 6 mL of methanol. A second elution (sample B in Figure 1) was achieved with 2 mL of 2% ammonium hydroxide in ethyl acetate. Finally, the rest (sample C in Figure 1) was eluted using a mixture of 2 mL of methylene chloride/isopropyl alcohol (80:20) with 2–10% (typical 5%) ammonium hydroxide. In many cases, the second step was omitted, and compounds of samples B and C were released from the matrix simultaneously by employing the third elutant. Each collected fraction was evaporated to dryness under a gentle stream of nitrogen at room temperature, and the residues were redissolved in 100 μL of running buffer. It is important to note that the column was never completely dried under full vacuum as requested by the manufacturer of the cartridge.[16]

Recovery. The recovery after sample pretreatment was determined by comparing MECC peak heights after extraction with peak heights obtained by direct injection of equal amounts of the drugs in buffer.

RESULTS AND DISCUSSION

As reported previously, drugs of abuse and/or their major metabolites can easily be analyzed in a borate/phosphate buffer (pH around 9.1) and with 75 mM SDS.[12] Without the addition of organic modifiers to the buffer (see ref 14 and also below), resolution is quite hampered in the presence of too many compounds, particularly with those which show rather high partitioning into the micelles. This can be circumvented via selective extraction of each class of compounds from a complex urine specimen. This, however, is a time-consuming approach and requires a rather large amount of urine (e.g. 5-mL aliquots per extraction). Therefore, a stepwise (digital) solid-phase extraction procedure from a single aliquot of urine was developed (Figure 1), a method which is similar to that reported by Dixit and Dixit for gas chromatographic analyses.[16] However, with the procedure given in that reference, poor results were obtained, particularly with the first elution of acidic and neutral compounds not providing good recovery of methaqualone and benzodiazepines. Using the recommended column drying under full vacuum, these compounds were mainly eluted with the subsequent methanol wash. However, without complete drying of the sorbent bed in the cleanup protocol, the expected results were obtained.

First, a three-step extraction procedure was investigated. The data presented in Figure 2 depict single-wavelength electropherograms obtained after elution with methylene chloride (panel A), ethyl acetate containing 2% NH_3 (panel B), and methylene chloride/isopropyl alcohol (80:20) with 2% NH_3 (panel C). A urine blank spiked with methaqualone, morphine, codeine, methadone, and heroin was employed (10 μg/mL each). The heroin in the standard solution used was partly decomposed to 6-acetylmorphine and morphine, this explaining the occurrence of a rather small heroin peak and the presence of 6-acetylmorphine (panel B). The first elution step recovered methaqualone, and the opioids were almost completely yielded in the second fraction. At this stage, the

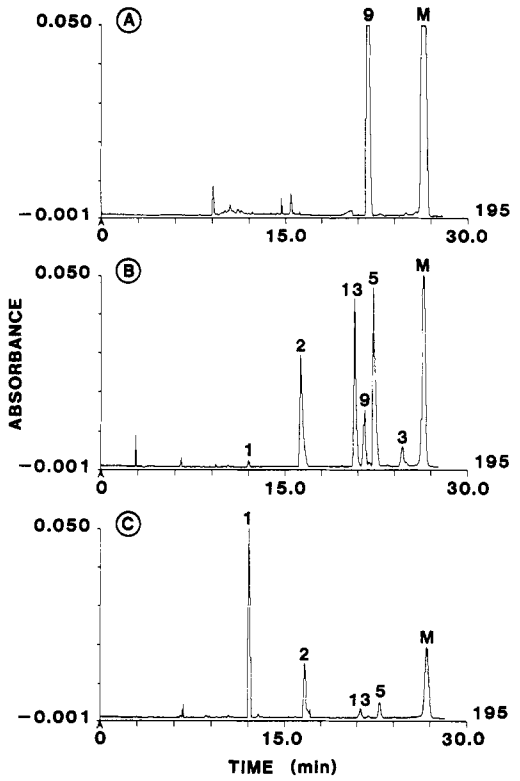

Figure 2. Single-wavelength (195-nm) MECC electropherograms obtained with the fractions of a three-step elution of a blank of urine spiked with different drugs. The applied voltage was a constant 20 kV, and the current was determined to be 79 μA. Key (for this and subsequent figures): (1) benzoylecgonine; (2) morphine; (3) heroin; (4) methamphetamine; (5) codeine; (6) amphetamine; (7) cocaine; (8) methadone; (9) methaqualone; (10) flunitrazepam; (11) oxazepam; (12) diazepam; (13) 6-acetylmorphine; (14) thiopental; (M) terminating zone containing compounds (including methadone) which are fully partitioned into the micelle. The sample identification is the same as given previously.[12]

metabolite of cocaine, benzoylecgonine, was still retained and was only eluted in the third step for which a mixture of methylene chloride/isopropyl alcohol containing 2% NH_3 was employed as elutant. This fraction was also found to contain a small amount of opioids. Methadone, whether recovered or not, cannot be analyzed in this experimental configuration, because it is eluting within the terminating peak (peak M in Figure 2) of compounds which are fully partitioned into the micelle. It is important to realize, that the three samples were obtained with the same aliquot of urine and had to be analyzed sequentially. Clean electropherograms were obtained with all three fractions (Figure 2), this showing that the adsorbed matrix compounds of the urine blank are efficiently removed in the wash and that the remaining compounds are not released during the drug elution steps.

Not surprisingly, reversal of the second and third elutions provided opioids and benzoylecgonine combined in fraction 2 and only very small amounts of these drugs were recovered in the following ethyl acetate elution (data not shown). The applicability of the simpler, two-step extraction procedure (with omission of the second elution step depicted in Figure 1) was further investigated. Single-wavelength and multiwavelength MECC data obtained with a urine blank which was spiked with thiopental, methaqualone, flunitrazepam, oxazepam, benzoylecgonine, morphine, codeine, and heroin (10 μg/mL each) are presented in Figure 3. Elution with methylene chloride provided a fraction containing the barbiturate (see also ref 11), methaqualone, and the two benzodiazepines (panels A and C) whereas the opioids and benzoylecgonine were yielded together with a subsequent elution employing a mixture of methylene chloride/isopropyl alcohol (80:20) containing 5% NH_3 (panels B and D). Although all compounds were found in the second fraction, there is a quite clear separation between the acidic/neutral and basic compounds, with methaqualone and oxazepam appearing more strongly in the second fraction than the other acidic and neutral substances. For that two-step extraction procedure and the conditions summarized under Materials and Methods, the detection limits of the first and second elution steps were determined to be slightly below and above 100 ng/mL, respectively. Recoveries were in the range 0.8–0.9, this corresponding to a 35–45-fold drug concentration during sample cleanup. For the basic drugs an even higher recovery was obtained when using 10% NH_3 in the elutant. This, however, occurred at the expense of increased elution of compounds from the matrix which in turn resulted in electropherograms which were somewhat more complex than those presented in Figure 3 (data not shown).

MECC data of a urine specimen of a hospitalized person which was (i) found to be markedly positive for cocaine, opiates, methaqualone, and methadone using EMIT-dau drug screening procedures and (ii) found to be EMIT-dau negative for amphetamines, barbiturates, and benzodiazepines, as well as (iii) found to be positive for cannabinoids employing a fluorescence polarization immunoassay, are depicted in Figure 4. With the two-step extraction procedure using methylene chloride as first elutant (panel A) and methylene chloride/isopropyl alcohol (80:20) containing 10% NH_3 as the second (panels B and C), the presence of methaqualone as well as benzoylecgonine and opioids, respectively, could be unambiguously confirmed. Peak assignment was achieved through comparison of retention times and absorption spectra of eluting peaks with those of computer-stored model runs (as discussed in detail previously[12]). The presence of 6-acetylmorphine (peak 13 in panels B and C) provides an indication of heroin consumption. Using calibration graphs based on peak heights (linear, four-point calibration with standards between 0.25 and 10 μg/mL), the concentrations of benzoylecgonine, methaqualone, morphine, and codeine in that patient's urine were estimated to be 11, 0.5, 7, and 1 μg/mL, respectively. The electropherograms of both fractions are more complex than those depicted in Figures 2 and 3. Obviously, metabolites and other drugs which remain to be identified are picked up as well.

The data presented in this paper demonstrate the applicability of simultaneous adsorption of different classes of drugs of abuse onto a mixed-mode phase followed by a stepwise (digital) release of drug fractions with multiple elutants and their analysis by MECC using a single-buffer system. With a two-step procedure, barbiturates, benzodiazepines, and methaqualone (first fraction), as well as opioids and cocaine metabolites (second fraction), are shown to extract with good recovery and detection limits which are in the same range as those of sensitive immunological screening methods (about 100 ng/mL). Using the same configuration, amphetamines are yielded equally well in the second step, provided the eluate is acidified prior to solvent evaporation and reconstitution (data not shown). Application of the two-step extraction procedure to a range of patient urine samples which tested positively by EMIT-dau tests for several classes of drugs, the presence of barbiturates, methaqualone, opioids, and cocaine metabolites could be unambiquously confirmed from a single aliquot of urine and without any sample hydrolysis or chemical derivatization. It is important to add that the confirmation of cannabinoids could not be achieved without hydrolyzing the urine specimen prior to extraction.[13] Hydrolysis of the patient urine (Figure 4) prior to extraction would permit the confirmation of cannabinoids. With this

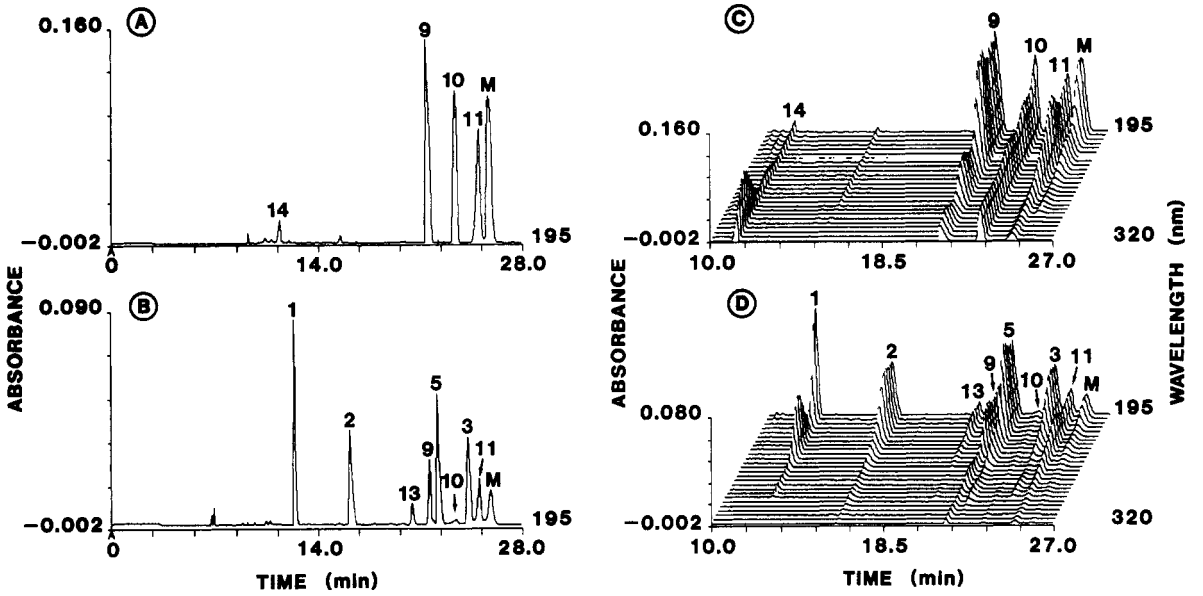

Figure 3. Multiwavelength and single-wavelength (195-nm) MECC electropherograms obtained by analyzing the fractions of a two-step elution process of a spiked urine blank. Other conditions and sample identification are the same as in Figure 2.

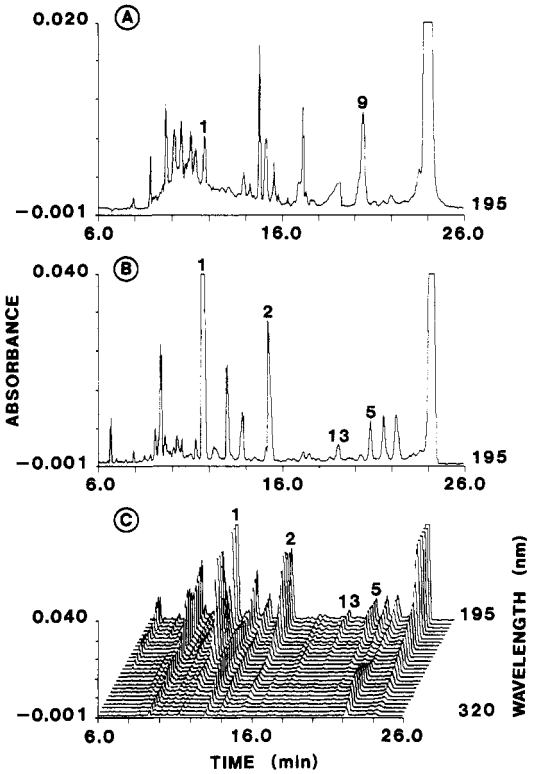

Figure 4. Multiwavelength and single-wavelength (195-nm) MECC electropherograms obtained with the fractions of a two-step extraction of a patient sample which tested positively for several classes of drugs. Other conditions and sample identification are the same as in Figure 2.

pretreatment, however, 6-acetylmorphine would be decomposed to morphine and the proof for heroin consumption would be lost. Therefore, confirmation of cannabinoids has to occur separately. The same applies for methadone which is found in the terminating micelle peak and therefore cannot be analyzed in the buffer system employed.

The MECC conditions used in our laboratory (75 mM SDS at a pH of about 9.1) revealed similar retention times for amphetamines and benzodiazepines, compounds which are recovered in separate fractions using the two-step extraction method. However, the MECC separation of amphetamine from methamphetamine,[12] as well as the separation of different benzodiazepines, could be improved. Reduction of buffer pH to 8.5 and/or changes in the SDS concentration to 50 or 100 mM did not provide any better data. Therefore, in analogy to the work of Weinberger and Lurie,[14] the addition of acetonitrile to the buffer was studied. Typical electropherograms obtained with a model mixture composed of benzoylecgonine, codeine, amphetamine, methamphetamine, diazepam, and methadone are presented in Figure 5. With the experimental configuration (instrument used, pH of 9.2, SDS concentration of 75 mM), the separation of the latter four substances is incomplete without the addition of acetonitrile (panel A). Dilution of that buffer with acetonitrile (5% in panel B; 10% in panel C) provided complete resolution and improved separation with increasing content of the organic buffer modifier. This was, however, at the expense of increased run times, a fact which was even more pronounced when the pH was lowered to 8.5 (panel D) or below (data not shown). In the instrument used and with a limitation of the analysis time to 50 min, no acetonitrile-containing buffer system was found which was capable of resolving all the illicit drugs investigated. Although the impact of the addition of other organic modifiers (such as 2-propanol and methanol[19]) remains to be investigated, it appears that stepwise extraction is very useful for drug confirmation with MECC because it permits the use of a buffer in which retention times can be kept reasonably low (here in the order of 25 min).

CONCLUSIONS

Multistep solid-phase extraction of different classes of drugs from a single aliquot of urine followed by sequential MECC analysis of each fraction in a given buffer provides a rapid and effective way to confirm drugs of abuse in human urine. For patient urines which tested positively for several classes of drugs using nonisotopic immunoassays, a two-step extraction scheme is shown to provide sufficiently simplified extracts suitable for rapid MECC confirmation of acidic and neutral drug substances (barbiturates, benzodiazepines, and methaqualone) in the first step, as well as basic compounds (opioids, amphetamines, and benzoylecgonine) in the second step. Neither hydrolysis nor sample derivatization are

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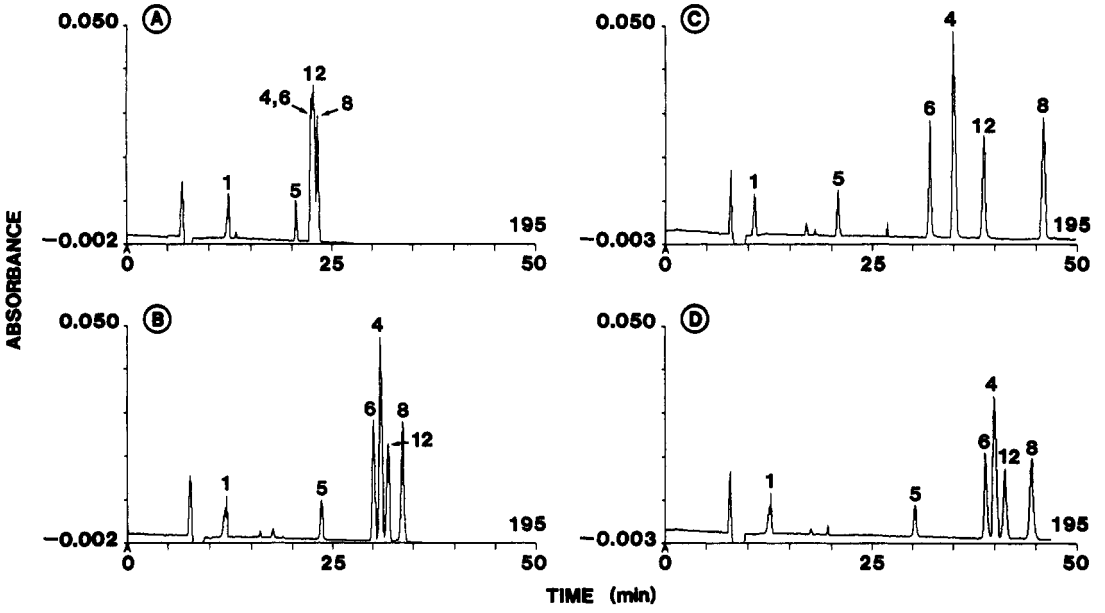

Figure 5. Single-wavelength (195-nm) electropherograms of a model mixture analyzed in borate/phosphate buffers with (A) 75 mM SDS and pH 9.2, (B) 71.25 mM SDS, 5% acetonitrile, and pH 9.2, (C) 67.5 mM SDS, 10% acetonitrile, and pH 9.2, and (D) 71.25 mM SDS, 5% acetonitrile, and pH 8.5. Other conditions and sample identification are the same as in Figure 2.

required or anticipated, this hindering the inclusion of the analysis of cannabinoids into this scheme[13] but permitting the unambiguous monitoring of the metabolite indicative for heroin consumption, 6-acetylmorphine (Figure 4). The multistep approach is more efficient compared to the sequential extraction of single classes of substances from multiple aliquots of urine prior to their analysis in optimized buffer systems. Its sensitivity is comparable to that obtained with the extraction procedures specific to single classes.[12] However, this technique is limited by the separability of the compounds of interest within a collected fraction. In special cases, single fractions from the multistep extraction scheme might have to be analyzed in different buffers.

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