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Performance of a pentafluorophenylpropyl stationary phase for the electrospray ionization high-performance liquid chromatography–mass spectrometry–mass spectrometry assay of cocaine and its metabolite ecgonine methyl ester in human urine

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Abstract

A pentafluorophenylpropyl (PFPP) bonded silica column has been used for the high-performance liquid chromatography–electrospray ionization–mass spectrometry–mass spectrometry assay (HPLC–ESI-MS–MS) of cocaine (COC) and its metabolite, ecgonine methyl ester (EME) in human urine. COC and EME were used as model basic solutes to demonstrate that a PFPP phase yields excellent results for the assay and validation of drugs in biological fluids. The assay was linear over three orders of magnitude (1.0–1000 ng/ml) and precision and accuracy of the assay was 4 and 15%, respectively. The limit of detection (LOD) for COC and EME was 1.6 and 2.8 pg on column, respectively. In addition, only a simple 1:10 dilution of the urine was necessary for the sample preparation procedure thus saving time on a laborious extraction step. The major advantage of the PFPP phase was the enhancement of the ESI-MS signal by providing good retention and good peak shape of COC and EME with a mobile phase of 90% acetonitrile. The MS signal for COC was a factor of 12 times greater on the PFPP phase than on the C_{18} phase.

Keywords: Stationary phases, LC; Cocaine; Ecgonine methyl ester; Pentafluorophenylpropyl

1. Introduction

The HPLC methods that exist for the analysis of drugs of abuse such as cocaine have been extensively reviewed [1]. Most of these methods use bonded C_8 or C_{18} phases for the analysis of these drugs and their metabolites in plasma [2], urine [3], hair [4] and fingernails [5]. However, since many drugs of abuse are basic with pK_a's greater than 8, high concentration of buffer salts and/or ion-pairing agents or counter-ions are often needed with these hydrophobic phases to achieve adequate peak shape and good retention with these columns [6]. The poor peak shape, often referred to as a tailing peak, is caused

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by the secondary interaction of the basic drug with the residual silanol groups [7]. To provide optimum signals in ESI-MS, high concentrations of buffer (>50 m*M*) and ion-pairing or ion-suppressing agents should be avoided [8]. Recently, high-purity silica has been produced which decreases the need for the use of mobile phase additives (buffers, ion-pair agents, etc.) to provide acceptable peak shape for the HPLC analysis of bases [9]. Even with the use of high-purity silica, C_8 or C_{18} bonded phases may not be the optimum phase when HPLC is interfaced to MS. Drugs such as cocaine and its more polar metabolites are hydrophilic; thus with typical reversed-phase columns (C_8 or C_{18}) low concentrations (<15%) of organic solvent or ion-pairing agents must be used to provide adequate retention of the solutes [10]. It has been reported that when the concentration of the organic solvent (MeOH, ACN, etc.) in the mobile phase is increased, the ESI-MS signal is increased because of more efficient desolvation in the ESI process [11,12].

Good HPLC and sample preparation procedures before an MS–MS analysis are needed in order to reduce the endogenous interferences in the sample that can cause ion-suppression in the ESI interface [13]. Ion suppression causes poor precision and diminished sensitivity in ESI-MS analyses. Analytes need retention times greater than 2 min to eliminate interferences that can cause ion suppression and less than 6 min in order to be cost efficient [14]. We have investigated a number of stationary phases and found that a pentafluorophenylpropyl (PFPP) stationary phase provided HPLC peaks with asymmetry factors near 1.0 and retention times between 2 and 6 min for basic drugs with the use of 90% acetonitrile in the mobile phase [15,16]. By using a high concentration of acetonitrile, the CN and PFPP stationary phases provided signal enhancements greater than a factor of 15 compared to a hydrophobic C_{18} stationary phase. However, the validation of an HPLC–MS–MS assay on a PFPP phase has not been demonstrated. We report the use of a PFPP stationary phase for the development and validation of an HPLC–ESI-MS–MS assay of cocaine and its metabolite, ecgonine methyl ester. Fig. 1 shows the structure of the PFPP stationary phase.

Cocaine, a major alkaloid from the coca plant is a common drug of abuse. A metabolite of cocaine, ecgonine methyl ester has a longer half-life than the parent drug and is often monitored by GC–MS analysis [17]. Although the GC–MS methods are sensitive and selective, the run times often take more than 15 min per sample. In addition, the GC–MS procedures require derivitization and tedious sample preparation methods to obtain adequate sensitivity and selectivity. Recently, Jeanville et al. developed an HPLC–MS–MS method that was sensitive and selective enough for the direct analysis of cocaine from human urine [18]. However, the gradient C_{18} HPLC method used with this method was unsuccessful in retaining the more polar ecgonine methyl ester ($k'<1$). Thus sufficient retention to prevent ionization suppression is an issue for the ESI-MS measurement of ecgonine methyl ester in complex matrices without extensive sample preparation. Due to its polar structure, ecgonine methyl ester is difficult to retain by C_{18} HPLC columns [19,20]. Since, our goal was to develop an HPLC–MS–MS assay on a PFPP stationary phase to show that the PFPP phase could have widespread use in the pharmaceutical industry, we developed an assay for COC and EME as model basic compounds. Since EME is the most difficult of the COC metabolites to retain by C_{18} columns, we chose EME and COC as our model solutes to determine if the validation of assays was possible on the PFPP stationary phase. For determination of the other metabolites of cocaine such as benzoylecgonine and cocaethylene see Refs. [1–5,18–20]. The PFPP phase should provide retention (t_R between 2 and 6 min) for COC and EME with a mobile phase that has a concentration of organic solvent of ~ 90%. The HPLC–MS–MS assay developed on the PFPP phase should be linear, accurate, precise, rugged and provide a limit of detection of at least 1 ng/ml of

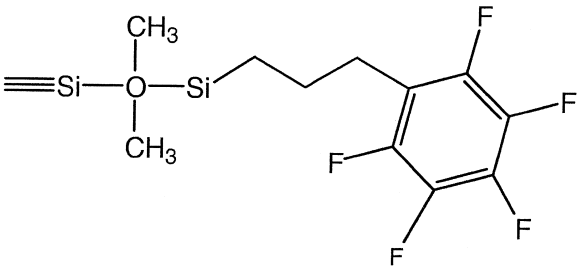
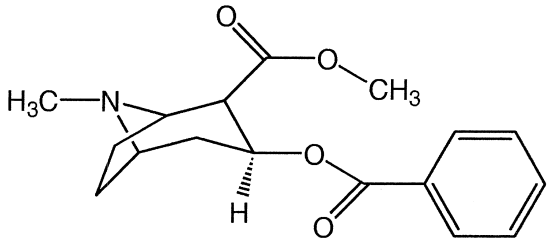


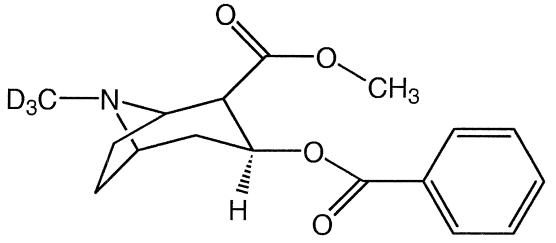
Fig. 1. Illustration of the PFPP stationary phase.

cocaine and ecgonine methyl ester. Structures of the solutes used in this investigation are shown in Fig. 2.

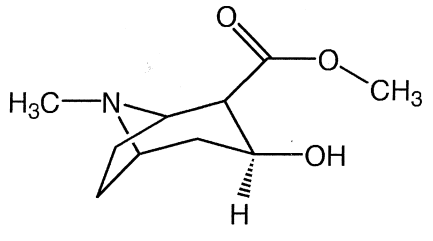
To our knowledge this is the first report that describes the use of a PFPP stationary phase in the validation of an HPLC–MS–MS assay. For COC and EME, the linearity, precision and accuracy of the method with the PFPP phase is reported along with qualitative ESI-MS signal comparisons to a C_{18} phase.



Cocaine



Cocaine-d_3



Ecgonine Methyl Ester

Fig. 2. Chemical structures of cocaine, ecgonine methyl ester and cocaine-d3.

2. Experimental

2.1. Reagents and standards

All compounds were obtained from Sigma Chemical (St. Louis, MO, USA). Standard stock solutions (1.0 mg/ml) were prepared by dissolving a weighed amount of the compounds in H_2O–MeOH (90:10 v/v%). The solutions were sonicated in an Ultrasonicating Bath 3200 (Bransonic, Danbury, CT, USA) for 10 min. The standards were stored at 4°C when not in use. HPLC reagents (J.T. Baker, Phillipsburg, NJ USA) were of HPLC grade or better. Ammonium formate was obtained from Spectrum Chemical Mfg. Corp. (Gardena, CA, USA) and was of >97% purity. Formic acid was obtained from Acros (New Jersey, USA) and was of 96% purity. The reagents for synthesis of the C_{18} stationary phase were obtained from Silar Laboratories (Scotia, New York, USA). The PFPP reagents were obtained from Gelest, Inc. (Tullytown, PA, USA). The reagents for the stationary phases were of >97% purity. All reagents were used without further purification.

2.2. HPLC columns

The PFPP and C_{18} HPLC columns were supplied by Restek Corporation (Bellefonte, PA, USA) and were 3.0 cm in length×2.1 mm I.D. The stationary phases were monofunctional. Columns contained packings of 5 μm particles with 60 Å pores. The phases were endcapped by bonding with trimethychlorolsilane. Both stationary phases are commercially available from Restek Corporation.

2.3. HPLC conditions and apparatus

Two Jasco 980 series pumps (Tokyo, Japan) equipped with a vacuum membrane degasser delivered the mobile phase at a flow rate of 0.6 ml/min. The mobile phase consisted of mixtures of acetonitrile and 5 m*M* ammonium formate adjusted to pH 3.0 with formic acid. A CTC LEAP Technologies HTS PAL autoinjector (Carrboro, NC, USA) injected 10 μl aliquots of the standards and samples onto the HPLC columns. The 30×2.1 mm

column void volume was estimated to be 0.13 min based on previous experiments [15].

2.4. Mass spectrometry

A Sciex API 3000 triple quadrupole mass spectrometer (PE-Sciex, Toronto, Ontario, Canada) equipped with a turbo ion spray interface (TISP) was used for the detection of analytes in the majority of this research. A Sciex API 2000 triple quadrupole mass spectrometer was also used to perform some of the initial collision induced dissociation (CID) experiments. Data was acquired in the positive ion mode with an ESI probe voltage of 5000 V. Nebulizer gas and curtain gas settings were 15 and 12 lb. in^{-2}, respectively. The TISP interface was operated at a temperature of 150°C and a drying gas setting of 7000 ml/min. LC2Tune version 1.4, Sample Control version 1.4 and Multiview version 1.4 were used for data acquisition and data analysis. Peak areas were calculated using extracted ion chromatograms with MacQuan version 1.6.

Mass spectrometer conditions (lens voltages, collision energy, etc.) were optimized by direct infusion of the standards into the TISP source. Multiple reaction monitoring (MRM) mode was used for data collection. Based on the CID spectra obtained in Fig. 3 and Ref. [18], the following MRM transitions were used for detection of analytes; 304.0 $m/z \rightarrow 182.0$ m/z for COC, 200.0 $m/z \rightarrow 182.0$ m/z for EME and 307.0 $m/z \rightarrow 185.0$ m/z for COC-d_3 (internal standard). Collision gas pressure with nitrogen was 2.2 mTorr. Collision energies were 28 eV for COC and COC-d_3. The collision energy used for EME was 26 eV. The electron multiplier was operated at 2100 V. The mass spectrometer was operated at unit mass resolution. Dwell times were approximately 0.2 s per ion monitored.

2.5. Calibration, quantitation and sample preparation

Real patient samples were obtained from the Forensic Sciences Institute at the University of Puerto Rico (San Juan, Puerto Rico) and stored at −5°C until use. Control human urine was obtained in-house from healthy, male volunteers. Urine samples were centrifuged at 2400 *g* for 10 min. To obtain quantitative numbers within the standard curve range, the samples were diluted 1:100 or 1:10 with H_2O before fortification with internal standard. Calibration and calibration verification samples were prepared from the stock solutions to cover the range 1.0–1000 ng/ml. The standards were fortified to give calibration points of 1.0, 5.0, 10, 50, 250, 500 and 1000 ng/ml and calibration curve verification points of 5.0, 50 and 500 ng/ml, respectively. Aliquots (0.5 ml) of the samples were then placed in a 96-well plate where approximately 100 ng (10 μl of 10 ng/μl) of the internal standard (COC-d_3) was added and the mixture was vortexed for 5 s. Calibration was performed by plotting peak area ratios of drug to internal standard against drug concentration. A linear $1/x^2$ fit was employed for all calibration curves. Concentrations for drug samples were calculated from the calibration fit.

Precision was expressed as coefficient of variation (%RSD). Accuracy was calculated as [(mean calculated concentration−nominal concentration)/nominal concentration]×100. The limit of quantitation (LOQ) was considered as the lowest concentration that can be discriminated from the baseline with a signal intensity ten times greater than the background level. Similarly, the limit of detection was determined based on a signal three times greater than the baseline level and reported as pg on column.

2.6. Intra- and inter-day assays

Variability studies were performed by injection of the calibration verification samples fortified with 5.0 ng/ml, 50 ng/ml and 500 ng/ml of COC and EME. Five samples at each concentration were analyzed on three separate occasions. Precision and accuracy were calculated as described above.

ACD Labs pK_a Calculator version 3.5 from Advanced Chemistry Development, Inc. (Toronto, Ontario, Canada) was used to calculate the pK_a's of the solutes.

3. Results and discussion

Molecular weight and pK_a information for COC, COC-d_3, and EME are listed in Table 1. The solutes

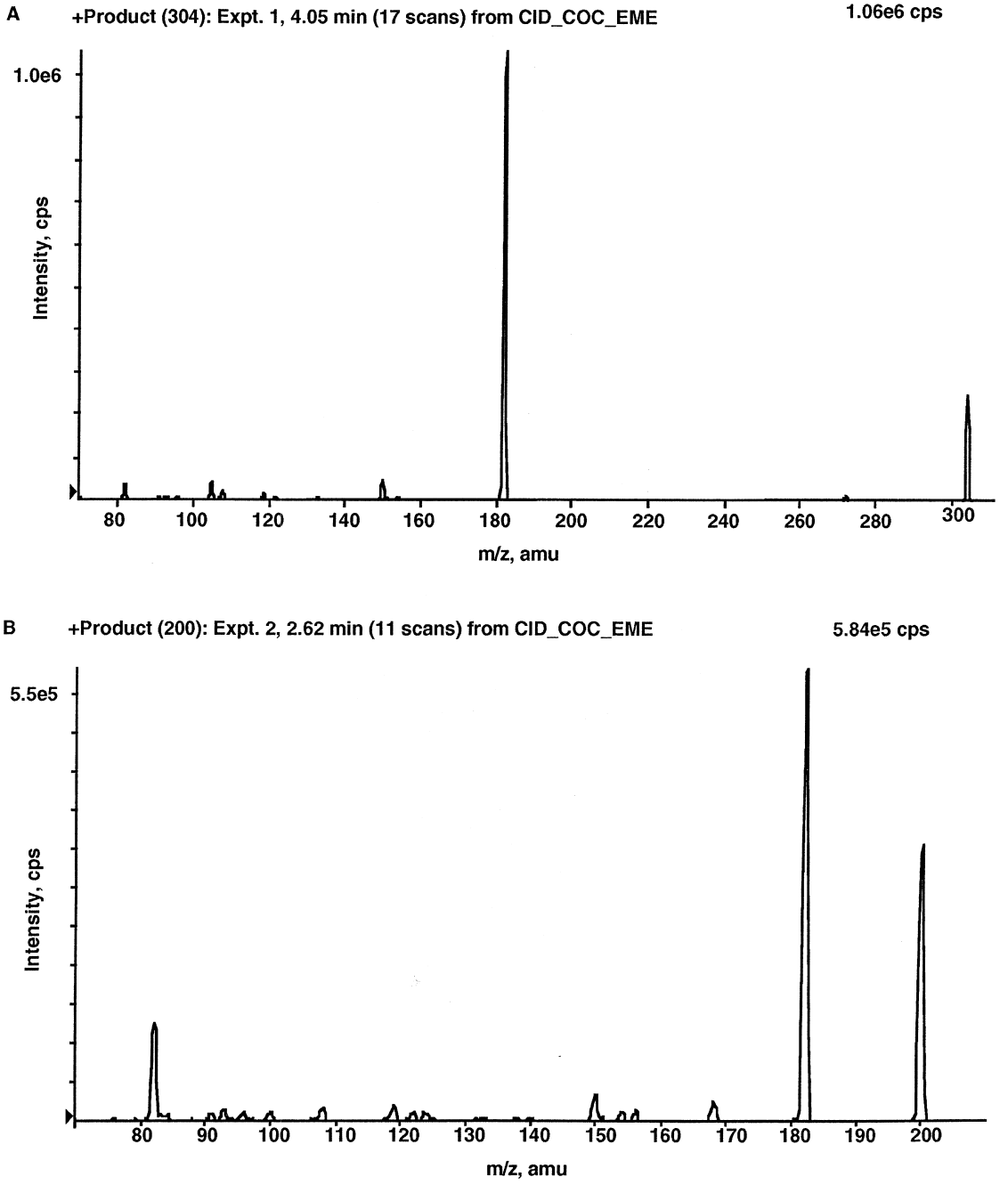


Fig. 3. Collision induced dissociation (CID) spectra for cocaine and ecgonine methyl ester (see Experimental section for CID conditions). (A) CID spectrum for the ESI/MS/MS analysis of cocaine. (B) CID spectrum for the ESI-MS–MS analysis of ecgonine methyl ester.

formed predominantly protonated molecules $[M+H]^+$ in the ESI source.

Fig. 3 shows the CID spectra of COC and EME. The MRM transitions (see Experimental) were chosen based on these spectra and previous studies [18]. In this manuscript we will briefly discuss the

Table 1
Major protonated molecular species observed from all solutes tested in positive ion ESI along with the drug class and pK_a information[a]

Drug	pK_a	Molecular weight
Cocaine	pK_a 8.7	303.4
Cocaine-d_3	pK_a 8.7	306.4
Ecgonine methyl ester	pK_1 9.3	199.1
	pK_2 14.2	

[a] Mobile phase consisted of mixtures of 90% acetonitrile and 5 m*M* ammonium formate, pH 3.0 at a flow rate of 0.6 ml/min. All pK_a information was obtained from ACD Labs pK_a calculator and correlate well with literature values [24].

CID mechanisms necessary to obtain an MRM transition of COC and EME. For a more detailed report on the fragmentation mechanisms of COC and COC-d_3 see Ref. [18]. COC forms fragments in the CID process to give a major product ion at m/z 182. This fragment ion is the ecgonidine methyl ester. In the CID process, EME decomposes to give the same major fragment as cocaine, ecgonidine methyl ester. This decomposition corresponds to a loss of H_2O from the parent ion of EME.

Ninety percent by volume of acetonitrile was chosen as the mobile phase based on previous HPLC/MS analyses with a PFPP stationary phase [16]. COC and the COC-d_3 had retention times of 3.9 min. EME had a retention time of 2.6 min. In this system a retention time of 2 min is equal to a k' of ≈14. Both COC and EME were eluted with good peak shape on the PFPP stationary phase.

3.1. Linearity

Calibration curves were constructed in the range of 1.0–1000 ng/ml for both COC and EME. Correlation coefficients for calibration curves of COC and EME were 0.995 and 0.993, respectively. In this range of calibration standards a slight deviation (≈10%) from linearity was detected near the upper limit of quantitation. Kebarle et. al. [21] have reported this deviation as due to the increased formation of dimers and trimers at higher concentrations thus the ESI-MS signal for the monomer is diminished.

Table 2
Accuracy and precision for the HPLC/ESI/MS/MS analysis of cocaine (COC) and ecgonine methyl ester (EME) in human urine

Drug	Nominal concentration (ng/ml)	Accuracy (%)[a]	Intra-assay Precision (%)[a]	Inter-assay Precision (%)[b]
COC	5.0	115	1.4	2.9
	50	110	1.2	3.0
	500	94	0.90	2.5
EME	5.0	115	1.9	3.1
	50	110	2.3	3.3
	500	92	1.7	2.5

[a] Mean of three experiments each performed in quintuple.
[b] Mean of calibration verification sample each performed in quintuple.

3.2. Accuracy and precision

In Table 2 data on the assay performance of COC and EME is presented. The intra-day and inter-day accuracy and precision were within 15% and 3.3% of nominal values, which are within the acceptable limits for pharmaceutical analyses [22]. It was found that the precision of the assay was improved if the calibration and calibration samples were calculated using an external standard calibration. Thus if no losses of sample occur during preparation or analysis, and the mass spectrometer performs consistently, than external standard is an acceptable form of HPLC–MS–MS calibration. However, COC-d3 was still used to verify instrument performance with each sample.

3.3. Specificity and selectivity

Assay selectivity is demonstrated by the absence of interfering peaks at the retention times of COC, COC-d_3 and EME in a blank urine sample. Fig. 4 depicts ion extracted chromatograms for the HPLC–MS–MS analysis of standards at 1.0 ng/ml (A) a blank sample (B) and a blank sample spiked with internal standard (C) to show that the COC-d_3 does not produce a response for COC or EME. As expected the COC and COC-d_3 are co-eluted but well separated from the EME in this HPLC system. Along with the selectivity shown by the analysis of a blank urine sample in Fig. 4. Fig. 5 shows a

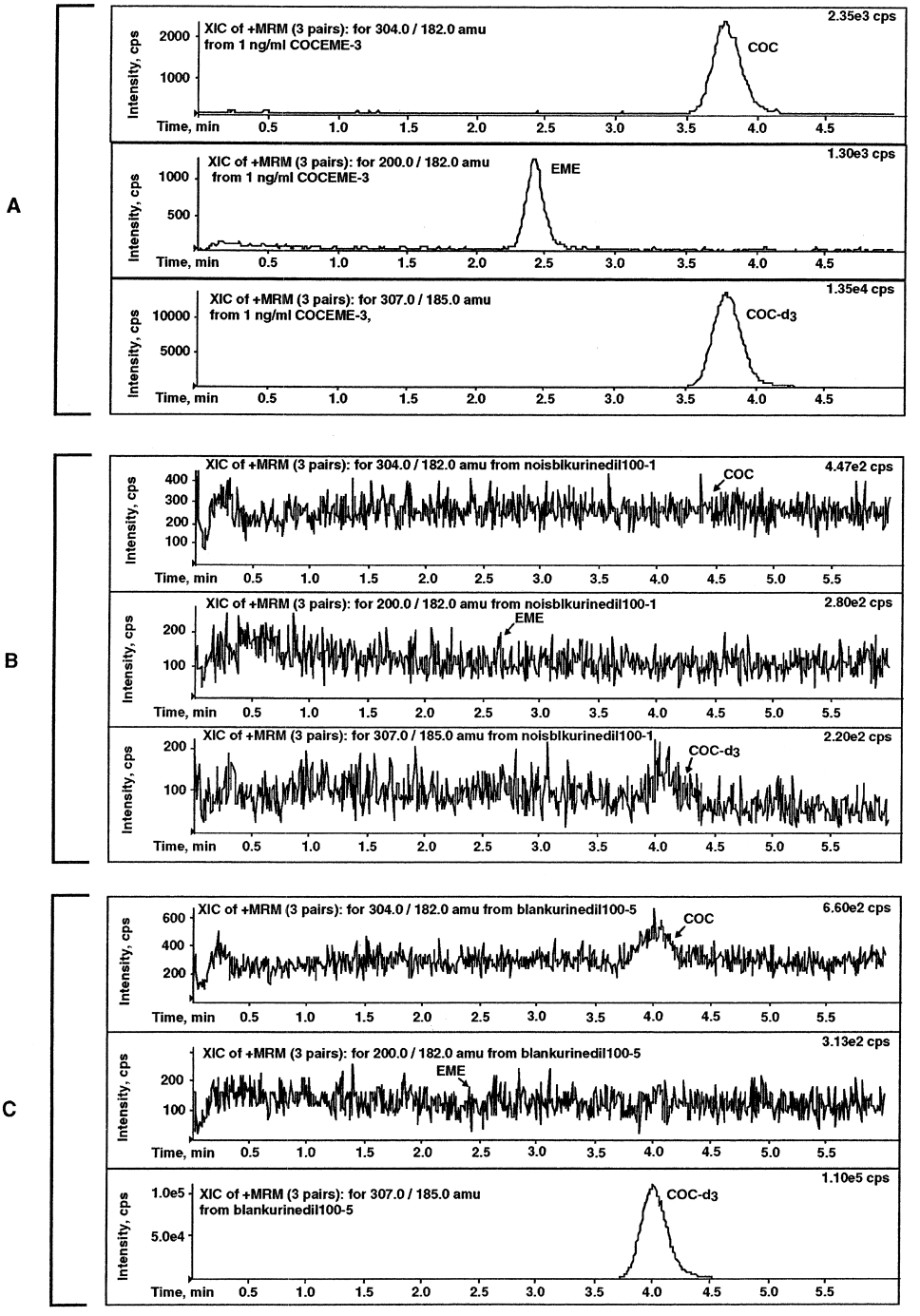


Fig. 4. Chromatograms for the HPLC–ESI-MS–MS analysis of cocaine and ecgonine methyl ester.(A) HPLC–ESI-MS–MS chromatogram from the analysis of a standard fortified at 1.0 ng/ml. (B) HPLC–ESI-MS–MS chromatogram from the analysis of a blank urine sample.(C) HPLC–ESI-MS–MS chromatogram from the analysis of a blank urine sample diluted by a factor of 10 and fortified with the internal standard, COC-d_3.

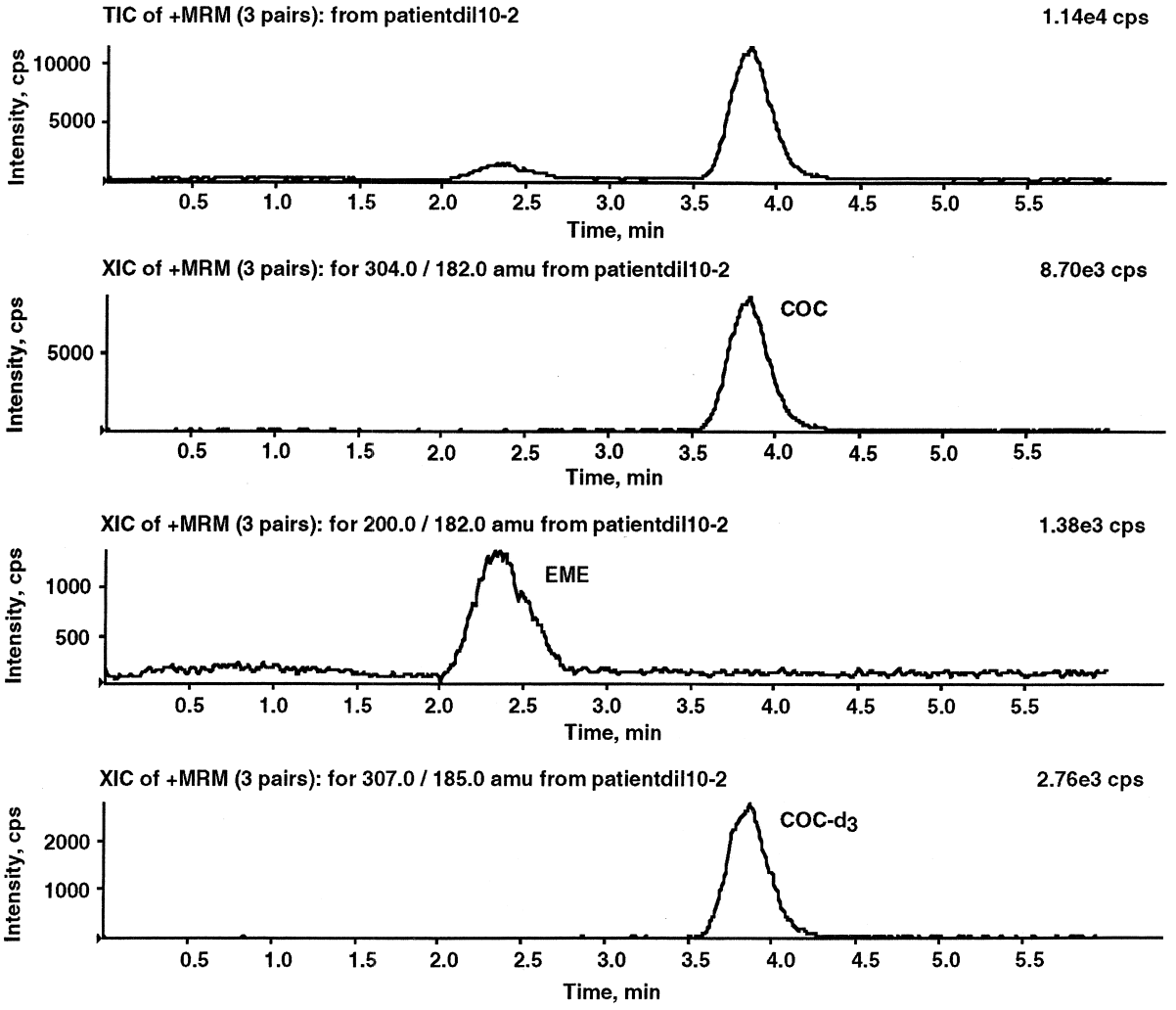


Fig. 5. HPLC–ESI-MS–MS chromatogram from the analysis of a patient sample obtained post-mortem and diluted 1:10.

chromatogram for the HPLC–MS–MS analysis of a patient's urine sample collected post-mortem. The slight response in the blank urine sample in the COC extracted ion chromatogram is from the COC-d3 internal standard that has less than 0.6% of non-deuterated COC. Obviously, this insignificant response did not largely affect the LOQ or any other portion of this assay. The calculated concentration for the patient sample was 44 and 24 ng/ml for COC and EME, respectively. As depicted in Fig. 5, the assay is selective and specific for the analysis of COC and EME.

3.4. Limits of detection and quantitation

The LOD for COC and EME as 1.6 and 2.8 pg on column, respectively. Taking into account the dilution factor of ten, the LOQ was 5.3 and 9.3 ng/ml for COC and EME, respectively. If the goal of this investigation was to achieve ultimate detection limits, than the LOD and LOQ could be improved by increasing the HPLC injection volume. In addition, it has been reported that when high concentrations of non-aqueous eluents are used, large volumes can be injected without loss of separation efficiency [23].

3.5. Method ruggedness

During this investigation, more than 200 injections were performed with the use of one PFPP column. These injections correspond to more than 20 h of continuous use of the column, in which 9000 column volumes of mobile phase were used.

3.6. Sensitivity of a PFPP phase conditions compared to a C_{18} phase

The MS signal was shown to be enhanced by a factor of 16 when the PFPP column was used in comparison to a C_{18} column [16]. Small, polar molecules such as EME can be especially difficult to retain on a C_{18} column. In this investigation, even when a 100% aqueous eluent was used, EME could not be retained on the C_{18} phase and in HPLC–ESI-MS, ionization suppression can occur if the solutes are not separated from endogenous compounds that are eluted near the void volume [14]. For the HPLC–ESI-MS–MS analysis of COC, the MS signal ($n=3$) was a factor of 12 greater on the PFPP phase than that obtained with the C_{18} phase. For the analysis of COC, 12% acetonitrile was required on the C_{18} phase to produce the same retention time (≈3.5 min) whereas 90% acetonitrile was used with the PFPP stationary phase. The increased MS signal is due to the more efficient desolvation that occurs with the use of acetonitrile as compared to water. Fig. 6 shows the chromatograms for the analysis of COC on a C_{18} and PFPP stationary phase. As shown in the chromatogram, better peak shape was also obtained on the PFPP phase compared to the C_{18} phase (Fig. 6).

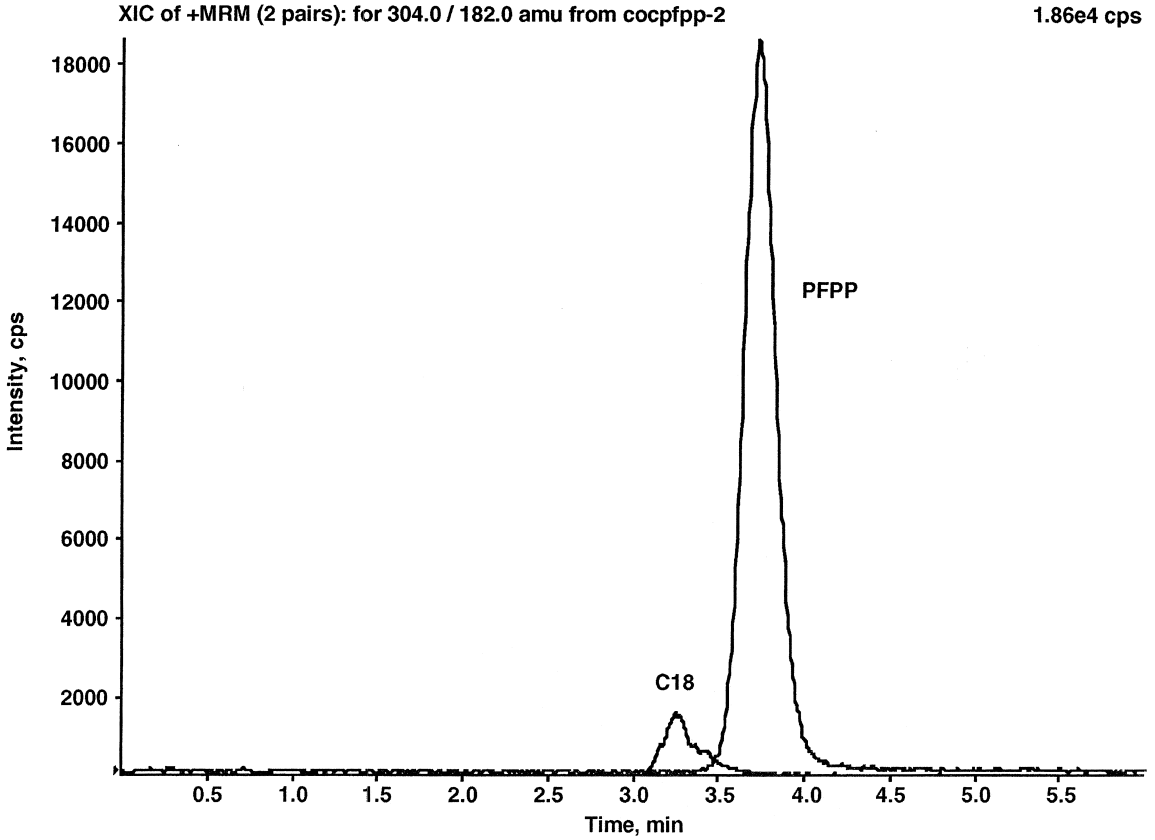


Fig. 6. The increase in the ESI-MS signal on a PFPP phase (90% acetonitrile) compared to a C_{18} phase (12% acetonitrile) for the HPLC–ESI-MS–MS analysis of cocaine (~100 pg on column) at 0.6 ml/min.

Because we prepared a new batch of mobile phase for the sensitivity experiments, slight changes in the retention times of COC and EME occurred. Since the desolvation is more efficient with the conditions used with the PFPP phase, the MS–MS baseline due to chemical noise is also increased by a factor of≈2.5 compared to that of the C_{18} phase. However, the enhancement in the MS signal is greater than the increase in the baseline noise which leads to a LOD greater than four times that on the PFPP stationary phase. The LOD for COC is 1.6 and 7.5 pg on column on the PFPP and C_{18} stationary phase, respectively. Since EME could not be retained on the C_{18} column, the LOD for EME could not be compared on the PFPP and C_{18} phase. Additional improvement of the MS signal is possible with the use of 100% acetonitrile in the mobile phase. However, retention times of COC and EME were not reproducible with 100% acetonitrile, possibly due to poor dissolution. Since the increase in the ESI-MS signal is due to an improvement in the desolvation process in the interface, operation of the mass spectrometer in the MS mode produces similar signal enhancements when the acetonitrile concentration in the mobile phase is increased. Details of increased ESI-MS signal in the MS mode are reported in Refs. [15,16].

4. Conclusions

The PFPP stationary phase was shown to be useful for the assay and validation of cocaine and its metabolite, ecgonine methyl ester in human urine by HPLC–ESI-MS–MS. The assay was precise, linear, rugged and accurate. The PFPP retains the basic drugs with the use of 90% acetonitrile in the mobile phase whereas a C_{18} stationary phase required only 12% acetonitrile in the mobile phase to obtain similar retention to the PFPP stationary phase. The high concentration of acetonitrile used with the PFPP phase improves the desolvation process in the ESI interface and thus produces lower detection limits. Due to improved desolvation and better peak shape, the detection limit of 1.6 pg on column for the HPLC–ESI-MS–MS analysis of cocaine on the PFPP stationary phase was four times better than the detection limit obtained on the C_{18} stationary phase. The PFPP stationary phase has been shown to give good retention and good peak shape for the analysis of cocaine and its metabolite, ecogine methyl ester. The PFPP phase will be useful for the low-level detection and assay validation of other basic drugs, metabolites and impurities in various matrices.

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