Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 833 (2006) 210–218
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous quantification of methamphetamine, cocaine, codeine, and metabolites in skin by positive chemical ionization gas chromatography–mass spectrometry

Wonkyung Yang [a], Allan J. Barnes [b], Mary G. Ripple [c], David R. Fowler [c], Edward J. Cone [d], Eric T. Moolchan [b], Heesun Chung [a], Marilyn A. Huestis [b,*]

[a] *National Institute of Scientific Investigation, 331-1 Shinwol 7-dong, Yangcheon-Gu, Seoul 158-707, Republic of Korea*
[b] *Chemistry and Drug Metabolism, National Institute on Drug Abuse, NIH, 5500 Nathan Shock Drive, Baltimore, MD 21224, USA*
[c] *Office of the Chief Medical Examiner, State of Maryland, 111 Penn Street, Baltimore, MD 21201, USA*
[d] *ConeChem Research, LLC, 441 Fairtree Drive, Severna Park, MD 21146, USA*

Received 21 November 2005; accepted 2 February 2006
Available online 24 February 2006

Abstract

A positive chemical ionization gas chromatography–mass spectrometric method was validated to simultaneously quantify drugs and metabolites in skin collected after controlled administration of methamphetamine, cocaine, and codeine. Calibration curves (2.5–100 ng/skin biopsy) for methamphetamine, amphetamine, cocaine, norcocaine, benzoylecgonine, cocaethylene, norcocaethylene, anhydroecgonine methyl ester, morphine, codeine, and 6-acetylmorphine (5–100 ng/skin biopsy for ecgonine methyl ester and ecgonine ethyl ester) exhibited correlation coefficients >0.999 and concentrations ±20% of target. Intra- and inter-run precisions were <10%. This procedure should be useful for postmortem analysis; data are included on drug concentrations in skin after controlled drug administration.

Keywords: Skin; Tissue; Biopsy; Methamphetamine; Cocaine; Codeine

1. Introduction

Blood and urine are the most common biological specimens for forensic testing; however, the investigation of alternate matrices as suitable samples to determine drug exposure is increasing. Drugs have been identified in sweat [1–3], hair [4–6], oral fluid [7–9], meconium [10], breast milk [11], and stratum corneum [12], but the disposition of drugs in skin biopsies has received only limited study. The measurement of drugs in alternative biological matrices may provide important pharmacokinetic information and help explain mechanisms of drug distribution throughout the body.

A 3-mm cylindrical piece of skin from the buttocks was collected by punch biopsy. Faergemann et al. [13–15] used a similar procedure to collect skin samples from subjects' backs. These punch biopsy samples contain epidermis, dermis, and fat tissue and weigh approximately 50 mg/skin biopsy. Skin is composed of two main layers, the outer and inner dermis. The top layer of the epidermis, stratum corneum, is composed of dead, flat skin cells that are shed about every 2 weeks. The dermis contains blood vessels, hair follicles, glands, and fat tissue. These glands produce sweat and sebum, an oily substance that prevents dehydration of the skin. An excretory duct from the sweat gland discharges sweat through pores onto the surface of the skin. Sebaceous glands deposit sebum onto hair follicles. Skin is constantly exposed to sweat and sebum and drugs in these secretions may be sequestered in skin and hair [12]. There also is evidence that after chronic exposure, lipophilic drugs may be stored in adipose tissue creating a drug depot and extending the exposure window [8,16,17].

Numerous methods employing derivatization, solid-phase extraction (SPE), and gas chromatography–mass spectrometry for the detection and measurement of single classes of drugs have been published [18–22], but few assays offer low detection and quantification limits for a broad range of drugs. Laboratories quantifying drugs of abuse have sensitive methods with

* Corresponding author. Tel.: +1 410 550 2711; fax: +1 410 550 2971.
E-mail address: mhuestis@intra.nida.nih.gov (M.A. Huestis).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.02.002

good analyte recovery, but traditionally have targeted only a single analyte or drug class. Due to the prevalence of polydrug use, there is a need in forensic testing for methods that simultaneously quantify multiple classes of drugs of abuse in limited specimen volume.

In this study, we developed and validated a method for the simultaneous determination of methamphetamine, cocaine, codeine, and metabolites in skin biopsies. This method employs dual silyl derivatization and positive chemical ionization gas chromatography–mass spectrometry (GC/PCI–MS) in support of our clinical research studies for the analysis of drugs in skin biopsies following subcutaneous cocaine and oral methamphetamine and codeine administration. We report results from more than 50 skin biopsies collected from 15 volunteers.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals were obtained from the following sources: (±)-methamphetamine, (±)-amphetamine, cocaine, norcocaine, benzoylecgonine, cocaethylene, norcocaethylene, anhydroecgonine methyl ester, ecgonine methyl ester, ecgonine ethyl ester, codeine, morphine, 6-acetylmorphine, (±)-methamphetamine-D_{11}, (±)-amphetamine-D_{10}, cocaine-D_3, benzoylecgonine-D_3, norcocaine-D_3, cocaethylene-D_3, ecgonine methyl ester-D_3, codeine-D_3, morphine-D_3, 6-acetylmorphine-D_3 (Cerilliant, Austin, TX, USA); *N*,*O*-bis(trimethyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) with 1% *tert*-butyldimethylchlorosilane (TBDMCS) (Pierce Chemical, Rockford, IL, USA). Solvents were HPLC grade and purchased from the following sources: methylene chloride, 2-propanol, and acetonitrile (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA) and methanol (Fischer Scientific, Fair Lawn, NJ, USA). Sodium acetate, acetic acid, ammonium hydroxide, and hydrochloric acid were A.C.S.-reagent grade and obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Filtration columns (RFV02F4P) and SPE columns (Clean Screen ZSDAU020) were obtained from United Chemical Technologies (Bristol, PA, USA).

2.2. Clinical application

Healthy volunteers ($N = 15$), with a history of stimulant and opiate use, provided informed consent to participate in this National Institute on Drug Abuse (NIDA) Institutional Review Board-approved study designed to investigate the excretion of drugs of abuse into alternate matrices. Participants resided on the closed clinical unit of the Intramural Research Program for up to 12 weeks. The first 2 weeks served as the wash-out phase for previously self-administered drugs. Low-dose administrations began in week 3; participants received four consecutive daily oral doses of methamphetamine–HCl (Desoxyn® Gradumet® 10 mg). In week 4, subjects received a subcutaneous injection of cocaine hydrochloride (75 mg/70 kg) three times within 1 week separated by a minimum of 48 h, and oral doses of codeine sulfate (60 mg/70 kg) on alternating days three times within the same week. Participants received placebo for 1 week, prior to high drug doses in week 7, following the same schedule and same routes. High dosages were 20 mg methamphetamine, 150 mg/70 kg cocaine, and 120 mg/70 kg codeine.

2.3. Human skin biopsy procedure

Physicians collected a 3-mm skin punch biopsy from the superficial skin layer of the gluteus maximus. Local anesthesia was applied prior to biopsy and a suture was used to close the incision, if necessary. The approximate weight of a 3-mm biopsy was 50 mg. The biopsy was repeated in a different area on study days 14, 30, 42, and 65; just prior to drug, following the last low dose, after placebo, and after the last high drug dose, respectively. There was a maximum of four biopsy specimens per participant. Biopsy specimens were stored at −20 °C until analysis.

2.4. Preparation of blank skin matrix

Human skin tissue was used for the development and validation of this analytical procedure. Single postmortem skin biopsy specimens (∼1 cm × 1 cm × 1 cm) were collected from an uninjured area on the mid-lower abdomen of individuals autopsied at the State of Maryland Medical Examiner's Office. These individuals had no investigative history of drug abuse, a postmortem interval less than 24 h, and were not suspected homicides. The skin surface was wiped free of debris and body fluids and the tissue was excised and stored in a labeled plastic container at −70 °C until transport to NIDA. Small skin specimens along with other organ biopsies are normally collected and held for posterity at autopsy. The portion of skin normally held for posterity was supplied to NIDA. Tissue was determined to be drug-free by GC/PCI–MS analysis prior to use as blank matrix in the preparation of calibrators and controls. Blank tissue was weighed and homogenized in 0.2 M sodium acetate buffer (pH 4.0) with a Kinematica Polytron probe, followed by sonication at room temperature to produce a tissue concentration of 100 mg/mL buffer. Five hundred microliters of the tissue homogenate was utilized for each calibrator or control. Homogenized skin tissues were stored at −20 °C until analysis.

2.5. Specimen preparation

Skin biopsy specimens (∼50 mg) were thawed and homogenized in 1 mL methanol. The probe was carefully cleaned with methanol (2 mL) between specimens, and these methanolic fractions were combined with the homogenate. Specimens were sonicated for 15–30 min at room temperature before evaporation under nitrogen at 40 °C. Extracts were reconstituted with 3 mL 2.0 M sodium acetate buffer pH 4.0.

2.6. Calibrators and controls

Stock solutions of methamphetamine, amphetamine, cocaine, norcocaine, benzoylecgonine, cocaethylene, norcocaethy-

lene, anhydroecgonine methyl ester, ecgonine methyl ester, ecgonine ethyl ester, codeine, morphine, and 6-acetylmorphine were diluted with acetonitrile to yield working calibrator solutions (250 and 5000 ng/mL).

Working calibrator solutions were added to certified drug-free skin tissue to generate daily calibration curves ranging from 2.5 to 100 ng/biopsy. Calibrators and controls were prepared from different commercially available drug lots.

Low-, medium-, and high-quality control samples were also prepared daily at concentrations of 6, 30, and 60 ng/biopsy for methamphetamine, cocaine, codeine, and metabolites from a working-quality control solution (600 ng/mL).

Each sample was fortified with 100 μL of a working internal standard solution (1000 ng/mL) containing (±)-methamphetamine-D_{11}, (±)-amphetamine-D_{10}, cocaine-D_3, benzoylecgonine-D_3, norcocaine-D_3, cocaethylene-D_3, ecgonine methyl ester-D_3, codeine-D_3, morphine-D_3, and 6-acetylmorphine-D_3. All solutions were prepared in acetonitrile and stored at −20 °C.

2.7. Extraction procedure

Specimens were analyzed for methamphetamine, cocaine, codeine, and metabolites using SPE according to a previously published procedure [3,23]. Extraction columns were conditioned with 1 mL elution solvent (methylene chloride:2-propanol:29.8% ammonium hydroxide, 80:20:2, v/v/v) followed by 3 mL methanol, 3 mL deionized water, and 1.5 mL 2.0 M sodium acetate buffer (pH 4.0). Prior to extraction, homogenized skin samples and prepared blank matrix (500 μL) were fortified with internal standards, diluted to 5 mL with additional acetate buffer and filtered through fritted reservoirs during centrifugation. After filtration, a liquid–liquid extraction with 1 mL of hexane was utilized to remove lipids. The supernatant (organic) was discarded and the aqueous fraction applied to preconditioned SPE columns and allowed to adsorb under gravity flow conditions. Columns were washed with deionized water (3 mL), 0.2N hydrochloric acid (1.5 mL), and methanol (3 mL) before aspiration under full vacuum for 5 min. Analytes were eluted with 4× 1 mL of elution solvent. All eluates were collected in conical glass centrifuge tubes.

2.8. Derivatization

Following SPE, 20 μL of MTBSTFA with 1% TBDMCS was added to each eluate. Eluates were evaporated under nitrogen in a water bath at 40 °C until dry. A 500 μL aliquot of acetonitrile was added, and tubes vortex mixed to recover drug from centrifuge tube walls. After evaporation to dryness, 20 μL acetonitrile was added, tubes were vortex mixed, centrifuged, and transferred to autosampler vials. A 20 μL aliquot of MTBSTFA with 1% TBDMCS was added to each vial. Vials were loosely capped and placed in a heat block at 80 °C for 15–20 min. After cooling, a 20 μL aliquot of BSTFA with 1% TMCS was added, vials were crimp capped and samples heated at 80 °C for 45 min. Two microliters of derivatized extract were injected for analysis under splitless GC/PCI–MS conditions.

2.9. Analytical conditions

Analysis of derivatized extracts was performed on an Agilent 6890 gas chromatograph and an Agilent 7673A automated liquid sampler interfaced with a mass-selective detector (Agilent 5973) utilizing positive chemical ionization. The flow controller was set to allow 20% of high-purity methane into the detector. Two microliters injections were made in the splitless mode with a purge time of 0.75 min. Injection port temperature was 250 °C. An initial oven temperature of 70 °C was held for 1.5 min followed by ramps of 23 °C/min to 170 °C and 21 °C/min to a final temperature of 310 °C that was held for 5 min. Separation of analytes was achieved in 17.5 min using an HP-5MS fused-silica capillary column (12 m × 0.2 mm I.D., 0.33 μm film thickness). Ultrapure grade helium was used as the carrier gas at a flow rate of 1.0 mL/min. Temperatures of the quadrupole, ion source, and mass-selective detector interface were 150, 250, and 285 °C, respectively. The mass-selective detector was operated in selective ion monitoring mode, with a dwell time of 10 ms for each analyte, and an electron multiplier setting of 400, relative to the daily tuning parameter. Three ions for each analyte and two ions for each internal standard were monitored. A list of target and qualifier ions can be found in Table 1.

2.10. Method validation criteria

The following criteria were used to evaluate the method: accuracy, precision (intra- and inter-run), sensitivity (limits of

Table 1
Analytes, internal standards, retention times, and target and qualifier ions for quantification of methamphetamine, cocaine, codeine, and metabolites in human skin biopsies by positive chemical ionization gas chromatography–mass spectrometry

Compounds	Retention time	Target ion	Q1[a]	Q2[a]
Amphetamine-D_{10}	5.88	162	244	
Amphetamine	5.89	158	234	250
Methamphetamine-D_{11}	6.81	136	217	
Methamphetamine	6.82	130	206	220
Ecgonine methyl ester-D_3	8.10	317	259	
Anhydroecgonine methyl ester[b]	5.85	182	210	150
Ecgonine methyl ester[b]	8.11	314	298	256
Ecgonine ethyl ester[b]	8.39	328	270	196
Cocaine-D_3	9.87	307	185	
Cocaine	9.88	304	182	272
Cocaethylene-D_3	10.10	321	199	
Cocaethylene	10.11	318	196	272
Norcocaine-D_3	10.26	365	243	
Norcocaine[c]	10.27	362	240	346
Norcocaethylene[c]	10.46	376	254	360
Codeine-D_3	10.87	285	375	
Codeine	10.88	282	372	356
Benzoylecgonine-D_3	11.08	407	285	
Benzoylecgonine	11.09	404	282	388
Morphine-D_3	11.93	459	475	
Morphine	11.94	456	472	382
6-Acetylmorphine-D_3	12.12	385	445	
6-Acetylmorphine	12.13	382	442	342

[a] Qualifier ions 1 and 2.
[b] Ecgonine methyl ester-D_3 utilized as internal standard.
[c] Norcocaine-D_3 used as internal standard.

quantification and detection), linearity, selectivity, extraction efficiency, and stability.

2.10.1. Accuracy and precision

Three quality control samples across the linear dynamic range of the assay (6, 30, 60 ng/biopsy) were evaluated to define accuracy and precision of the method. Intra-assay data were assessed by comparing analyte concentrations of low, medium, and high control samples ($N = 10$), and inter-assay data ($N = 30$) were determined from five analytical batches (10 samples from one batch and five replicates at each level in four additional batches). Data were evaluated using one-way analysis of variance with day as the grouping variable. Accuracy, expressed as a percentage, was calculated by taking the difference between mean calculated and target concentrations and precision expressed as percent relative standard deviation (%R.S.D.).

2.10.2. Sensitivity

The sensitivity of the assay for each analyte was evaluated by determining limits of detection (LOD) and quantification (LOQ). The LOD was defined as the lowest analyte concentration with acceptable peak shape, chromatographic resolution, retention time (±2% of target), qualifier ion ratios (±25% of average calibrator ratios), and a signal-to-noise ratio (determined by peak height) of at least 3:1. The LOQ was defined as the lowest calibrator that met LOD criteria and had analyte concentrations within ±20% of target values.

2.10.3. Linearity

Daily calibration curves were prepared in singlate with equal weighting in each analytical batch. Linearity was determined by the method of least squares and expressed as the correlation coefficient (r^2). The concentrations of each calibrator were within ±20% of the target concentration when calculated against the six-point calibration curve (2.5, 5, 10, 25, 50, and 100 ng/biopsy).

2.10.4. Selectivity

Selectivity is defined as the ability of the method to identify and quantify analyte with or without the presence of other endogenous or exogenous constituents. Blank skin tissue samples ($N = 6$) were prepared as described to check for the absence of potentially interfering endogenous compounds. In addition, blank matrix with internal standard added was evaluated in each analytical batch to verify that there were no interferences from the internal standard or from endogenous compounds. Potential exogenous interferents could include structurally similar compounds, metabolites, co-administered drugs, or over the counter medications. To assess potential interferences, low-quality control samples (6 ng/biopsy) were spiked individually to contain 10,000 ng/biopsy of acetaminophen, caffeine, nicotine, phenypropanolamine, ephedrine, pseudoephedrine, 3,4-methyledioxymethamphetamine, dextromethorphan, phentermine, fenfluramine 3,4-methyledioxyamphetamine, methadone, 3,4-methyledioxyethylamphetamine, hydrocodone, hydromorphone, oxycodone, oxymorphone, pentazocine hydrochloride, phencyclidine, clonidine, and diphenhydramine hydrochloride.

2.10.5. Extraction efficiency

Recovery or extraction efficiency was determined for each analyte at two concentrations (10 and 100 ng/biopsy) by adding internal standard to a set of spiked samples ($N = 5$) after SPE but before evaporation and to a second set ($N = 5$) before extraction. All samples were derivatized and analyzed by GC/PCI–MS. Extraction efficiency (%) was calculated by comparing mean concentrations of extracted control samples to mean concentrations of samples spiked with internal standards after SPE.

2.10.6. Stability

To evaluate stability of analytes, a set of low and high (6 and 60 ng/biopsy) unextracted quality control samples ($N = 5$) were subjected to three freeze–thaw cycles prior to extraction. Each freeze–thaw cycle consisted of freezing at −20 °C for 24 h, and thawing at ambient temperature before refreezing. Analyte concentrations of stability samples ($N = 5$) were compared to freshly prepared and analyzed quality control samples. Stability of derivatized extracts was also examined. GC autosampler vials containing low-, medium-, and high-quality control samples were stored at room temperature for up to 48 h following initial analysis. Samples were re-injected and concentrations compared to initial quality control results.

3. Results

3.1. Method development

The single-step trimethylsilyl (TMS) BSTFA derivatization scheme utilized in our laboratory for the simultaneous analysis of cocaine and opiates is robust. Methamphetamine and amphetamine have become important target analytes in our controlled drug administration studies, requiring an assay that concurrently monitors all three drug classes. However, when single-step TMS derivatization was employed for the analysis of amphetamine and methamphetamine, recovery and sensitivity were erratic due to evaporative losses incurred during sample concentration. Traditionally, quantification of volatile compounds has been difficult for precisely these reasons [24]. Addition of aqueous HCl to the eluate has been employed to induce hydrochloride formation and reduce volatility [25]. An impaired ability to detect basic drugs at low concentrations following the addition of trace amounts of acids and salts also has been reported [26]. The addition of aqueous HCl can require higher evaporation temperatures or longer evaporation times that can result in analyte loss. Initially, our goal was to utilize a single-step derivatization scheme employing MTBSTFA. It was anticipated that the molecular mass increase associated with *tert*-butyldimethylsilyl (TBDMS) derivatization (114 versus 72 *m/z* per derivative) might increase the uniqueness or specificity of mass fragments, which could increase specificity and sensitivity. Unfortunately, the volatility problem with the amphetamine analogs was not alleviated with single-step TBDMS derivatization.

Table 2
Accuracy and precision data for quantification of methamphetamine, cocaine, codeine, and metabolites in human skin biopsies

Analyte	Target (ng/biopsy)	Intra-assay ($N=10$)				Inter-assay ($N=30$)			
		Mean (ng/biopsy)	S.D.	Accuracy (%)	Precision (%)	Mean (ng/biopsy)	S.D.	Accuracy (%)	Precision (%)
Methamphetamine	6	5.9	0.10	99.1	1.8	6.0	0.16	99.9	2.7
	30	28.7	1.10	95.6	3.8	29.6	1.15	98.5	3.9
	60	59.5	1.06	99.2	1.8	59.7	1.07	99.5	1.8
Amphetamine	6	5.9	0.24	98.3	4.0	6.0	0.21	100.6	3.5
	30	29.8	1.09	99.4	3.7	30.3	0.89	100.9	2.9
	60	60.1	0.83	100.2	1.4	60.5	0.72	100.8	1.2
Cocaine	6	5.6	0.28	93.5	4.9	6.0	0.36	99.4	6.0
	30	29.8	1.16	99.3	3.9	30.7	1.62	102.4	5.3
	60	58.2	1.49	97.1	2.6	59.5	1.43	99.1	2.4
Norcocaine	6	6.1	0.25	101.1	4.2	6.0	0.17	99.8	2.9
	30	29.5	0.65	98.2	2.2	30.2	0.88	100.7	2.9
	60	58.3	1.46	97.1	2.5	59.8	1.54	99.7	2.6
Benzoylecgonine	6	5.8	0.21	96.5	3.6	5.9	0.22	98.0	3.7
	30	28.8	0.85	96.1	3.0	29.4	1.20	98.0	4.1
	60	58.4	1.02	97.3	1.8	59.0	1.03	98.3	1.7
Cocaethylene	6	6.0	0.25	99.3	4.2	6.0	0.17	100.5	2.9
	30	30.6	0.66	101.9	2.2	30.7	0.71	102.3	2.3
	60	59.5	1.15	99.2	1.9	60.2	1.06	100.4	1.8
Norcocaethylene	6	6.2	0.32	102.7	5.2	6.0	0.28	99.7	4.6
	30	28.0	0.80	93.5	2.9	28.5	1.36	94.9	4.8
	60	55.6	2.48	92.7	4.5	58.2	2.66	97.0	4.6
Anhydroecgonine methyl ester	6	5.9	0.17	98.9	2.8	6.0	0.15	99.3	2.5
	30	30.3	0.74	100.9	2.4	30.2	0.86	100.7	2.8
	60	59.8	1.64	99.6	2.7	59.9	1.26	99.9	2.1
Ecgonine methyl ester	6	5.9	0.14	98.1	2.3	5.9	0.18	99.2	3.1
	30	29.7	0.71	99.1	2.4	30.1	0.74	100.5	2.5
	60	59.2	1.33	98.6	2.2	59.7	1.28	99.4	2.2
Ecgonine ethyl ester	6	5.9	0.09	98.3	1.5	5.9	0.17	98.1	3.0
	30	28.5	1.32	94.9	4.7	28.2	0.98	94.0	3.5
	60	58.4	1.01	97.3	1.7	58.6	2.59	97.7	4.4
Codeine	6	6.3	0.41	104.7	6.5	6.1	0.41	102.2	6.7
	30	30.4	1.16	101.5	3.8	30.7	1.58	102.4	5.1
	60	59.2	1.09	98.7	1.8	60.9	1.70	101.5	2.8
Morphine	6	5.3	0.16	89.1	3.0	5.7	0.42	95.3	7.3
	30	27.2	1.91	90.8	7.0	28.9	2.13	96.4	7.4
	60	49.4	0.69	82.4	1.4	56.3	5.18	93.8	9.2
6-Acetylmorphine	6	5.9	0.33	97.9	5.6	5.9	0.30	98.3	5.0
	30	30.3	1.83	101.0	6.0	30.2	1.31	100.8	4.3
	60	57.4	2.26	95.7	3.9	58.2	2.59	97.1	4.4

In our experiments, we investigated the initial addition of MTBSTFA to the extraction eluate prior to sample concentration to reduce evaporative loss. To test this hypothesis, MTBSTFA was added to neat standards prior to evaporation. After drying down, samples were reconstituted with acetonitrile and placed in a heating block before injection. Ions for underivatized methamphetamine and amphetamine and their corresponding TBDMS derivatives were monitored. Interestingly, following TBDMS stabilization our GC/PCI–MS method only detected underivatized amphetamines with poor chromatographic characteristics, indicating that our improved recovery could not be attributed to a mass increase from silyl donation. One possible mechanism for this stabilization may be salt formation similar to what occurs with post-extraction addition of inorganic acids like HCl. Addition of MTBSTFA to samples after SPE and before evaporation was found to greatly reduce volatility losses of the amphetamines. In this report, we describe the validation of a combined SPE GC/PCI–MS method employing dual silyl derivatization with a post-SPE stabilization step to monitor amphetamine, cocaine, and opiate analytes in skin biopsies.

Table 3
Limits of detection and quantification and calibration curves ($N = 5$) for drugs and metabolites in human skin biopsies

Analyte	Range (ng/biopsy)	Regression equation of calibrators[a]	Correlation coefficient[a]	LOD[b] (ng/biopsy)
Amphetamine	2.5–100	$y = 0.041\ (0.001)x - 0.035\ (0.026)$	0.9997 (0.0004)	1.25
Methamphetamine	2.5–100	$y = 0.052\ (0.003)x + 0.081\ (0.048)$	0.9995 (0.0001)	2.5
Cocaine	2.5–100	$y = 0.034\ (0.008)x + 0.030\ (0.022)$	0.9996 (0.0003)	2.5
Norcocaine	2.5–100	$y = 0.046\ (0.002)x + 0.019\ (0.019)$	0.9991 (0.0008)	1.25
Benzoylecgonine	2.5–100	$y = 0.044\ (0.006)x + 0.022\ (0.056)$	0.9997 (0.0002)	2.5
Cocaethylene	2.5–100	$y = 0.045\ (0.001)x + 0.039\ (0.023)$	0.9997 (0.0003)	2.5
Norcocaethylene	2.5–100	$y = 0.051\ (0.002)x - 0.012\ (0.019)$	0.9997 (0.0003)	1.25
Anhydroecgonine methyl ester	2.5–100	$y = 0.052\ (0.004)x + 0.022\ (0.026)$	0.9998 (0.0001)	2.5
Ecgonine methyl ester	5–100	$y = 0.031\ (0.001)x + 0.078\ (0.013)$	0.9998 (0.0002)	5
Ecgonine ethyl ester	5–100	$y = 0.041\ (0.001)x + 0.003\ (0.035)$	0.9998 (0.0002)	5
Codeine	2.5–100	$y = 0.056\ (0.036)x + 0.005\ (0.026)$	0.9998 (0.0001)	2.5
Morphine	2.5–100	$y = 0.060\ (0.042)x + 0.002\ (0.056)$	0.9996 (0.0004)	2.5
6-Acetylmorphine	2.5–100	$y = 0.077\ (0.044)x + 0.057\ (0.064)$	0.9995 (0.0006)	2.5

[a] Mean value (standard deviation).
[b] Limit of detection.

3.2. Method validation

3.2.1. Accuracy and precision

Accuracy and precision evaluated over the method's linear range at 6, 30, and 60 ng/biopsy are presented in Table 2. Intra-assay precision was assessed for 10 replicates of the low-, medium-, and high-quality control samples. Inter-assay precision was determined for 30 samples analyzed in five analytical batches. Intra-assay accuracy (% difference between mean and target concentrations) and precision (%R.S.D.) for all analytes ranged from 82.4 to 104.7 and 1.4 to 7.0%, respectively. Inter-assay accuracy and precision ranged from 93.8 to 102.4 and 1.2 to 9.2%, respectively. Data evaluated using a single-factor analysis of variance with day as the grouping variable showed significant differences between days ($p = 0.05$). However, when comparing daily mean analyte concentrations, only the low-codeine control (10.3%) and the high-morphine control (14.3%) exceeded a mean percentage difference of more than 7.0%.

3.2.2. Linearity and sensitivity

Characteristic calibration data, including analyte regression equations ($N = 5$), are described in Table 3. The calibration range encompasses the expected concentrations to be found in biopsy samples, with calibration responses for all compounds linear to 100 ng/biopsy. Correlation coefficients (r^2) for all analytes exceeded 0.999. LOQ for extracted methamphetamine, cocaine, codeine, and metabolites were 2.5 ng/biopsy with the exception of ecgonine methyl ester and ecgonine ethyl ester, which were 5.0 ng/biopsy. The sensitivity (LOD) of the method was assessed by analyzing replicate samples ($N = 3$) of decreasing analyte concentrations in blank tissue. Amphetamine, norcocaine, and norcocaethylene satisfied all chromatographic criteria with acceptable ion ratios for the 1.25 ng/biopsy; all other analytes had LOD quantifications that were equal to their LOQ.

3.2.3. Selectivity

Twenty-one potential interfering compounds were spiked (10,000 ng/biopsy) into low quality control samples (6 ng/biopsy) to evaluate selectivity. Sixteen compounds (dextromethorphan, pseudoephedrine, phentermine, fenfluramine, ephedrine, phencyclidine, oxycodone, oxymorphone, 3,4-methylenedioxymethamphetamine, diphenhydramine, 3,4-methylenedioxyethylamphetamine, 3,4-methylenedioxyamphetamine, methadone, caffeine, nicotine, and acetaminophen) did not interfere with quantification of any of the analytes of interest. Lower concentrations of interferents were evaluated if the higher concentration produced unacceptable chromatography, ion ratios or quantification. Four compounds (phenylpropanolamine, pentazocine, hydromorphone, hydrocodone) did not interfere at spiked concentrations of 1000 ng/biopsy. Quantification of norcocaethylene in the presence of clonidine (1000 ng/biopsy) was within 20% of target, and had an acceptable Q1 ratio, but showed an obvious interference (distorted peak shape) in the second qualifier ion ratio. No analyte interferences were detected during analysis of blank skin biopsies from six different sources. In addition, during each analytical run, the absence of native analyte ions in the negative sample (blank biopsy and internal standard) demonstrated that potential impurities in the internal standard did not contribute to measured concentrations of analytes of interest.

Table 4
Extraction efficiency (%) of drugs and metabolites in human skin biopsies ($N = 5$)

Analyte	Low (10 ng/biopsy)	High (100 ng/biopsy)
Amphetamine	91.3	96.1
Methamphetamine	94.6	96.8
Cocaine	104.6	100.9
Norcocaine	99.6	97.6
Benzoylecgonine	90.5	95.8
Cocaethylene	98.1	97.3
Norcocaethylene	95.0	97.1
Anhydroecgonine methyl ester	93.7	94.3
Ecgonine methyl ester	96.0	94.9
Ecgonine ethyl ester	96.3	93.5
Codeine	90.2	94.7
Morphine	122.6	116.2
6-Acetylmorphine	100.6	96.3

Table 5
Freeze–thaw stability of methamphetamine, cocaine, codeine, and metabolites in human skin biopsies ($N = 5$)

Analyte	Mean 6 ng control	Mean freeze–thaw	S.D.	CV	Mean % accuracy	Mean 60 ng control	Mean freeze–thaw	S.D.	CV	Mean % accuracy
Amphetamine	6.3	6.1	0.04	0.6	97.4	61.9	61.2	0.56	0.9	98.9
Methamphetamine	6.0	6.0	0.05	0.7	100.0	61.0	61.7	1.21	2.0	101.2
Cocaine	6.4	6.5	0.06	0.9	102.5	61.9	60.3	1.35	2.2	97.3
Norcocaine	6.3	6.3	0.14	2.2	99.0	60.9	59.9	2.00	3.3	98.4
Benzoylecgonine	6.2	6.3	0.10	1.5	100.7	61.5	60.8	1.14	1.9	98.9
Cocaethylene	6.4	6.5	0.22	3.5	100.7	62.9	59.6	1.06	1.8	94.7
Norcocaethylene	6.4	6.3	0.11	1.7	99.3	60.3	59.4	1.51	2.5	98.5
Anhydroecgonine methyl ester	6.0	6.2	0.25	4.1	103.7	62.1	82.6	1.55	1.9	133.0
Ecgonine methyl ester	6.3	6.3	0.16	2.6	100.3	61.2	78.9	4.15	5.3	129.0
Ecgonine ethyl ester	5.9	6.1	0.07	1.1	103.1	61.2	70.2	2.57	3.7	114.8
Codeine	6.6	6.7	0.17	2.6	100.8	63.2	62.0	0.82	1.3	98.2
Morphine	4.9	5.4	0.10	1.9	111.1	60.7	60.9	0.33	0.5	100.5
6-Acetylmorphine	6.2	6.4	0.12	1.9	102.5	59.3	60.8	0.90	1.5	102.5

3.2.4. Extraction efficiency

Internal standards were added after solid-phase extraction of samples ($N = 5$) at two concentrations (10 and 100 ng/biopsy). Mean analyte concentrations were compared to extracted controls ($N = 5$) to evaluate extraction efficiency. Extraction efficiencies, expressed as percent recovery, for all analytes at all concentrations were between 90 and 123% (Table 4).

3.2.5. Stability

Analyte concentrations were stable in unextracted tissue samples ($N = 5$) following three freeze–thaw cycles. Mean analyte concentrations of these stability samples (6 and 60 ng/biopsy) were compared to routine extracted controls ($N = 5$) with results presented in Table 5. Comparisons of drug concentrations to routine controls were reported as ±14% of target for all analytes, except for ecgonine methyl ester (±29%) and anhydroecgonine methyl ester (±33%). Stability of derivatized extracts in capped GC autosampler vials at room temperature was assessed after 48 h. Derivatized low and high control samples ($N = 6$) were stable, with acceptable quantifications within 20% of target.

3.3. Application to skin biopsy specimens

This validated method was applied to 55 skin biopsies collected from 15 volunteers after controlled administration of methamphetamine, cocaine, and codeine. The linear dynamic range of the assay exceeded expected tissue concentrations. Due to the invasive nature of specimen collection, a maximum of four biopsy samples were collected per participant. In order to maximize data collected, one biopsy was taken prior to drug administration and one following the completion of the low, placebo, and high-dosing regimen. Representative extracted ion chromatograms for (a) a blank skin biopsy containing only internal standards, (b) 10 ng/biopsy calibrator, and (c) a representative positive specimen are shown in Fig. 1.

The first biopsy was collected 14 days after residing on the closed clinical unit and just prior to controlled drug administration. Drug concentrations in this biopsy reflect previously self-administered drugs. Specimens were analyzed for 13 analytes, including 6-acetylmorphine, potentially present from prior heroin use. Cocaine was the only analyte detected in 2 of 15 biopsies (3.0 and 4.7 ng/biopsy).

The biopsy collected following the completion of the low doses reflects drug content at 12, 4, and 1 day after administration of the last methamphetamine, cocaine, and codeine doses, respectively. Methamphetamine and amphetamine were not detected in any of the 15 biopsy specimens collected after the low doses. Four days after the last low cocaine dose (total dose 225 mg/70 kg), only one specimen was positive for cocaine at a concentration of 2.7 ng/biopsy. This same individual was also positive (3.1 ng/biopsy) following the high cocaine doses (total dose 450 mg/70 kg). The two individuals that had been positive for cocaine in skin prior to controlled administration were negative following the low cocaine doses. Codeine was measurable in seven specimens, ranging from 2.7 to 7.0 ng/biopsy. No morphine was detected. The timing of the biopsy collection, 4 and 1 day after the last cocaine and codeine doses, made detection of these analytes more likely than detection of methamphetamine.

After the placebo session, biopsy specimens were collected from 13 volunteers. Methamphetamine (2.6 ng/biopsy) and trace amounts of amphetamine (>LOD) were the only analytes detected in a single skin specimen. Interestingly, the last of four consecutive 10 mg methamphetamine doses was administered 25 days prior to collection and the prior biopsy was negative for methamphetamine and amphetamine collected 12 days after the last low dose. No skin biopsies were positive for methamphetamine and amphetamine 12 days after 40 and 80 mg of methamphetamine were administered.

Twelve biopsies were collected 1 day after the high-dose session. Four biopsies were positive for codeine (3.0–4.5 ng/biopsy) and one for codeine and cocaine at 7.8 and 3.1 ng/biopsy, respectively. All participants positive after the high-dose session ($N = 5$) were also positive for the same analytes after the low doses. Despite this fact, there was no statistically significant relationship between drug dose and concentrations in skin biopsy specimens.

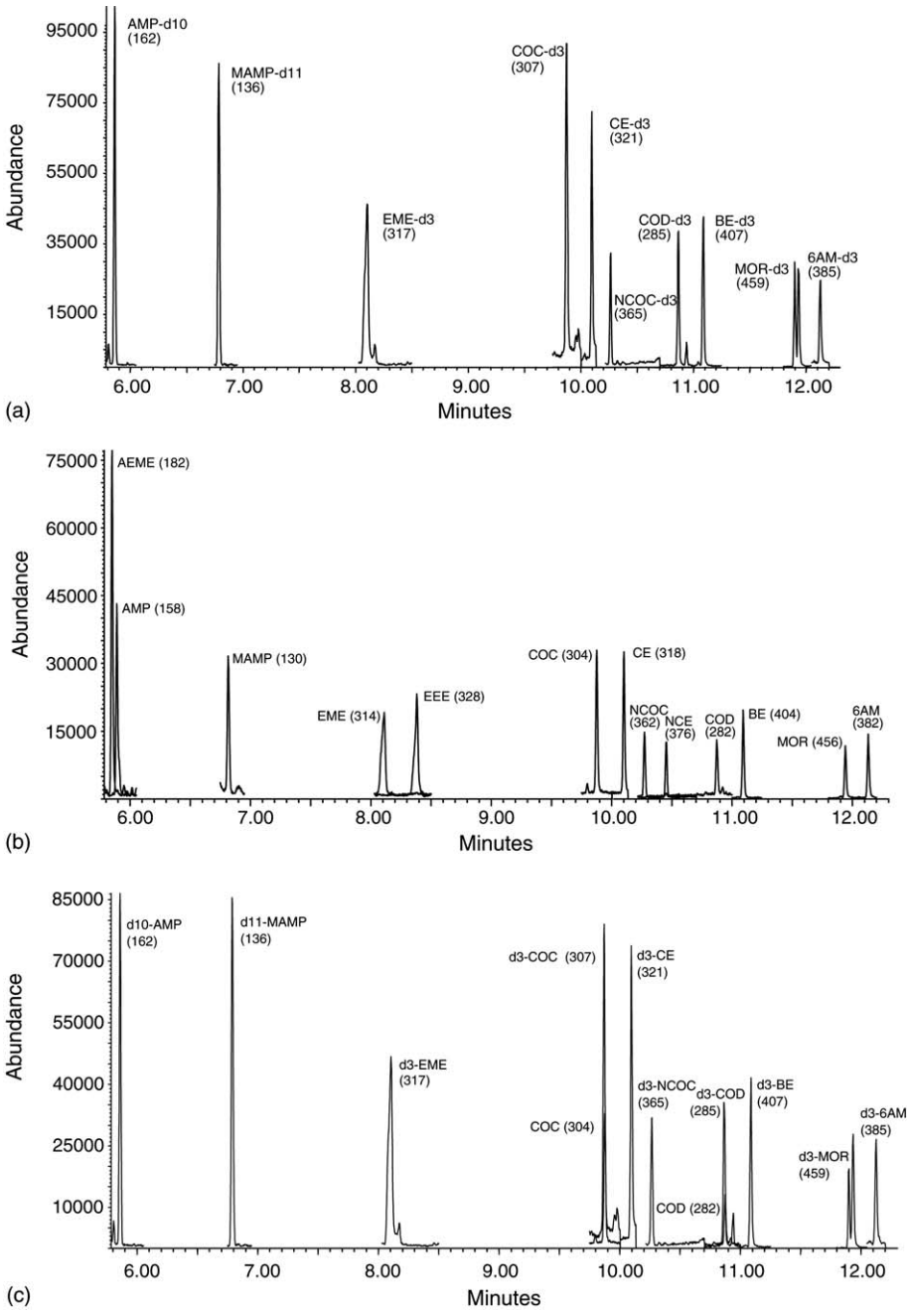

Fig. 1. Extracted ion chromatograms for (a) blank skin biopsy fortified with deuterated internal standards, (b) 10 ng calibration standard, and (c) skin biopsy containing 2.7 ng/biopsy cocaine and 7.0 ng/biopsy codeine. AEME, anhydroecgonine methyl ester; MAMP, methamphetamine; AMP, amphetamine; EME, ecgonine methyl ester; EEE, ecgonine ethyl ester; COC, cocaine; NCOC, norcocaine; CE, cocaethylene; NCE, norcocaethylene; BE, benzoylecgonine; COD, codeine; MOR, morphine; 6AM, 6-acetylmorphine.

4. Discussion

This article describes an analytical procedure with suitable selectivity, accuracy, precision, and recovery for the simultaneous quantification of methamphetamine, cocaine, codeine, and metabolites in skin biopsy specimens by positive chemical ionization GC/MS. The development and validation of an analytical method that can quantify exposure to multiple drug classes and metabolites offers several advantages including the cost of reagents, supplies, and labor. Furthermore, savings are realized in the required numbers of expensive instruments and capillary columns and other consumables that might be required for analysis, and of the time needed to interchange equipment between different analytical assays. In addition, multiple data are produced in the most time-efficient manner. The most important advantage is the ability to maximize information in cases with limited specimen availability.

Only 13% of skin biopsies ($N = 2$) collected from 15 individuals, at least 2 weeks after the last self-administered illicit drug,

were positive for cocaine at low concentrations (<5 ng/biopsy). Skin biopsies from these same two individuals were negative for cocaine at the method LOQ of 2.5 ng/biopsy after subcutaneous administration of 225 and 450 mg/70 kg of cocaine hydrochloride. An extended detection time of up to 10 days for cocaine following heavy chronic use was reported in urine and saliva [8]. The authors suggested that the long detection time was most likely due to depots of cocaine in deep body compartments. Data from the present study suggest that cocaine stored in skin and fat following self-administration of illicit cocaine was rarely measurable after 2 weeks. Also, all biopsies collected prior to controlled drug administration were negative for codeine, morphine, and 6-acetylmorphine, despite self-reported use of heroin for as long as 23 years and as frequently as 15 times in the last month prior to protocol admission.

Excluding biopsies taken prior to drug administration, codeine was the primary analyte detected in 30% of biopsy specimens ($N = 40$) collected after methamphetamine, cocaine, and codeine dosing. However, it is likely that these results were influenced by the proximity of specimen collection (1 day) to drug administration. Codeine concentrations ranged from 2.7 to 7.8 ng/biopsy after oral administration of up to 360 mg/70 kg codeine sulfate. Four days after subcutaneous administration of a total of 450 mg/70 kg cocaine hydrochloride, only 5% of skin biopsies were positive for cocaine (2.7–4.7 ng/biopsy), and only a single skin specimen was positive after oral administration of up to 80 mg of methamphetamine. No other analytes were detected at our limits of quantification.

We have developed and validated a rugged, sensitive, and specific method for medical examiner/coroner laboratories performing postmortem analyses to simultaneously and accurately quantify methamphetamine, cocaine, codeine, and metabolites in a tissue matrix. Furthermore, this method should be useful for further investigation of the disposition of drugs into and out of skin and the relative contribution of drugs stored in skin to drug measured in plasma, sweat, and hair. These data call into question the relative contribution of drugs in skin to drug concentrations in sweat and hair. In addition, drug concentrations in skin may not be a major contributor to the extended excretion half-lives observed after chronic, heavy drug use. An alternative explanation could be that drugs are stored in skin at concentrations below the LOQ of this method, and that the large surface area of skin represents a large depot of drug that is slowly released over time, supporting the described extended excretion times.

Acknowledgments

This research was supported by the Intramural Research Program of the National Institute on Drug Abuse, National Institutes of Health. We would also like to thank the post-doctoral Fellowship Program of Korean Science & Engineering Foundation for support.

References

[1] E.J. Cone, M.J. Hillsgrove, A.J. Jenkins, R.M. Keenan, W.D. Darwin, J. Anal. Toxicol. 18 (1994) 298.
[2] P. Kintz, A. Tracqui, C. Jamey, P. Mangin, J. Anal. Toxicol. 20 (1996) 197.
[3] M.A. Huestis, J.M. Oyler, E.J. Cone, A.T. Wstadik, D. Schoendorfer, R.E. Joseph Jr., J. Chromatogr. B 733 (1999) 247.
[4] C. Girod, C. Staub, Forensic Sci. Int. 107 (2000) 261.
[5] E.J. Cone, D. Yousefnejad, W.D. Darwin, T. Maquire, J. Anal. Toxicol. 15 (1991) 250.
[6] L. Skender, V. Karacic, I. Brcic, A. Bagaric, Forensic Sci. Int. 125 (2002) 120.
[7] A.J. Jenkins, J.M. Oyler, E.J. Cone, J. Anal. Toxicol. 19 (1995) 359.
[8] E.J. Cone, W.W. Weddington Jr., J. Anal. Toxicol. 13 (1989) 65.
[9] I. Kim, A.J. Barnes, J.M. Oyler, R. Schepers, R.E. Joseph Jr., E.J. Cone, D. Lafko, E.T. Moolchan, M.A. Huestis, Clin. Chem. 48 (2002) 1486.
[10] J. Oyler, W.D. Darwin, K.L. Preston, P. Suess, E.J. Cone, J. Anal. Toxicol. 20 (1996) 453.
[11] P.H. Dickson, A. Lind, P. Studts, H.C. Nipper, M. Makoid, D. Therkildsen, J. Forensic Sci. 39 (1994) 207.
[12] R.E. Joseph, J.M. Oyler, A.T. Wstadik, C. Ohuoha, E.J. Cone, J. Anal. Toxicol. 22 (1998) 6.
[13] J. Faergemann, H. Zehender, J. Denouel, L. Millerioux, Acta Derm. Venereol. 73 (1993) 305.
[14] J. Faergemann, H. Laufen, Clin. Exp. Dermatol. 18 (1993) 102.
[15] J. Faergemann, H. Zehender, T. Jones, I. Maibach, Acta Derm. Venereol. 71 (1990) 322.
[16] J.A. Levisky, D.L. Bowerman, W.W. Jenkins, S.B. Karch, Forensic Sci. Int. 110 (2000) 35.
[17] G.L. Henderson, Forensic Sci. Int. 63 (1993) 19.
[18] R.A. Gustafson, E.T. Moolchan, A. Barnes, B. Levine, M.A. Huestis, J. Chromatogr. B 798 (2003) 145.
[19] B.D. Paul, S. Lalani, T. Bosy, A.J. Jacobs, M.A. Huestis, Biomed. Chromatogr. 19 (2005) 677.
[20] I. Kim, D. Darwin, M. Huestis, J. Chromatogr. B 814 (2005) 233.
[21] C. Meadway, S. George, R. Braithwaite, Forensic Sci. Int. 127 (2002) 136.
[22] F.T. Peters, T. Kraemer, H.H. Maurer, Clin. Chem. 48 (2002) 1472.
[23] J.M. Oyler, E.J. Cone, R.E. Joseph Jr., E.T. Moolchan, M.A. Huestis, Clin. Chem. 48 (2002) 1703.
[24] B.K. Gan, D. Baugh, R.H. Liu, A.S. Walia, J. Forensic Sci. 36 (1991) 1331.
[25] D.E. Blandford, P.R.E. Desjardins, Clin. Chem. 40 (1994) 145.
[26] T. Kraemer, H.H. Maurer, J. Chromatogr. B 713 (1998) 163.