The influence of the sample matrix on LC-MS/MS method development and analytical performance

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Chapter 5.2

Application of sweat patch screening for 16 drugs and metabolites using a fast and highly selective LC-MS/MS method

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ABSTRACT

To facilitate the monitoring of drug abuse by patients, a method was developed and validated for fast and highly selective screening for amphetamine, methamphetamine, MDMA, MDA, MDEA, methylphenidate, cocaine, benzoylecgonine, morphine, codeine, heroin, 6-MAM, methadone, EDDP, nicotine and cotinine in PharmCheck™ sweat patches. The analysis of sweat patches would provide a non invasive alternative matrix to urine or blood samples.

The sweat patches were extracted during vigorous shaking for 10 minutes with 1.5 mL ammonium formate 20 mmol/L pH7 and methanol (50:50 % v/v). The extracts were cleaned up by filtering through Whatman mini-Uniprep™ syringeless filter vials prior to injection. The method uses a single injection to detect and confirm all 16 drugs and metabolites within 9.6 minutes.

The validated substances have a linear range of 3.0–300 ng/patch, except for nicotine which has a linear range of 30–3,750 ng/patch. Stabilities of all substances in worn sweat patches were validated at room temperature for 7 days and as a processed sample in the auto-sampler at 10°C for 5 days. Only heroin was unstable, with high individual variability and reported bias and CV of respectively -30.6% and 22.1% in worn sweat patches at room temperature. The monitoring of ion ratios was added to the validation criteria. This resulted in analytical cut-off concentrations of 3.0 ng/patch and 60 ng/patch for nicotine with validated qualifier/quantifier ratios. All analytical cut-off concentrations were lower than the cut-off concentrations proposed by the SAMHSA (Substance Abuse and Mental Health Services Administration).

The method uses validated cut-off concentrations, qualifier/quantifier ratios and a simple extraction without extensive sample treatment for the analysis of 16 drugs and metabolites with a runtime of 9.6 minutes. This method was successfully applied for the analysis of 96 worn sweat patches to monitor patients for drug abuse. The results provided the physician or health care professional with information about drug abuse and could be used to improve patient care with patient specific therapy.

INTRODUCTION

In order to monitor patients for drug abuse various tools can be employed. Information about drug abuse can be obtained through self-reports and assessment by health care professionals [1, 2]. This approach can provide the health care professional with valuable information. An analytical method to screen for drugs in sweat patches is a useful tool to complement information and to confirm or refute suspicions about drug abuse. Urine and blood are mostly used to detect drug abuse over a period of days. Information about drug abuse over longer periods of time can be provided by segmental analysis of hair strands, so that single exposure can be distinguished from long-term exposure [3-7]. Sweat patches are a non-invasive alternative to provide information about drug abuse during a period up to approximately one week [8]. Besides the non-invasive nature of sweat patches they also show attempts of adulteration, whereas a urine sample is easily tampered with through in vivo and in vitro adulteration and urine substitution [9].

The PharmCheck™ sweat patch consists of an absorptive pad covered with a non-occlusive membrane. Ingested drugs are excreted through sweat glands in the human skin and absorbed by the sweat patches.

Worn sweat patches can be sent to the laboratory for analysis. For 10 of the 16 substances, cut-off concentrations have been set by the the Substance Abuse and Mental Health Services Administration (SAMHSA) [10]. Several methods have been described for the analysis of multiple substances in sweat patches. To obtain information about drug abuse, the analysis of multiple substances with the use of one extraction and analysis method is desirable. Due to the complexity of developing a multi substance analysis method many articles describe the analysis of just one group of substances [8, 11-21]. Most analyses are performed using GC-MS and require extensive sample preparation with, for example, solid phase extraction (SPE) and derivatization [11, 13, 16, 21, 22]. Only one article described sweat patch analysis of multiple substances performed with LC-MS/MS, but this analysis still used an extensive sample preparation with SPE [23].

The Commission of the European Communities has established a directive concerning the performance of analytical methods and the interpretation of results [24]. The directive describes maximum permitted tolerances for relative ion intensities and is based on the relative intensity of the qualifier fragment compared to the quantifier fragment. The directive states that a qualifier, which is more than 10-fold less sensitive than the quantifier fragment,

is allowed to have a deviation of 50% from the standardized ratio for a positive confirmation of the detected drug.

Considering the fact that the quantifier mass transition is almost always the most intense fragment and the qualifier mass transition the second most intense fragment, it is imperative that the less sensitive qualifier mass transition has been proven to be reliable at the validated cut-off concentration. In that case the sensitivity of the method needs to be sufficient for both the quantifier and qualifier mass transitions.

Although external contamination seems unlikely, several investigations have shown that external contamination of the worn sweat patches can cause false positives. Since passive exposure can arise from any amount of drug, setting cut-off levels other than those based on analytical performance are unlikely to be effective for the distinction between drug use and passive exposure [25]. These investigations have exposed the disadvantages and in some cases unreliability of sweat patch analysis to assess drug abuse. Several court cases have been dismissed due to concerns with environmental contamination influencing the patch results [25]. Therefore, it can be concluded that sweat patches are not able to indisputably prove the use of drugs. However, the non-invasive character of sweat patch testing can provide a useful complementary tool for physicians and health care professionals to evaluate possible drug abuse.

In order to be able to monitor drug abuse, an extraction and analysis method for PharmCheck™ sweat patches was developed and validated for the most widely used drugs and their metabolites. The following substances were included in the method: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), methylphenidate, cocaine, benzoylecgonine, morphine, codeine, heroin, 6-monoacteylmorphine (6-MAM), methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP, metabolite of methadone), nicotine and cotinine.

MATERIALS AND METHODS

Chemicals and reagents

Separate reference solutions containing amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxy-

ethylamphetamine (MDEA), methylphenidate, cocaine, benzoylecgonine, morphine, codeine, heroin, 6-monoacteylmorphine (6-MAM), methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), nicotine and cotinine of 1.0 mg/mL in methanol were used. Cotinine was purchased from Cerilliant (Round Rock, Texas, USA). All other drugs were purchased from Lipomed (Arlesheim, Switzerland). Deuterated IS were used for all drugs. Lipomed reference solutions were used for 6-MAM-D_3, benzoylecgonine-D_3, cocaine-D_3, codeine-D_3, EDDP-D_3, heroin-D_3, MDA-D_5, MDEA-D_5, MDMA-D_3, methadone-D_3, methamphetamine-D_5, morphine-D_3. Cerilliant reference solutions were used for cotinine-D_3, methylphenidate-D_9, nicotine-D_4 and amphetamine-D_{10}. Analytical grade methanol, 25% ammonia and dichloromethane (DCM) were purchased from Merck (Darmstadt, Germany). Purified water was prepared by a Milli-Q Integral system (Billerica, Massachusetts, USA). Ammonium formate was purchased from Acros (Geel, Belgium).

Equipment and conditions

All experiments were performed on an Agilent 6460A (Santa Clara, CA, USA) triple quadrupole LC-MS/MS system, with an Agilent 1200 series combined LC system. The LC-MS/MS settings used were based on an earlier developed method [3]. The Agilent 6460A mass selective detector was operated in heated electrospray positive ionisation mode and performed dynamic multiple reaction monitoring (DMRM) with unit mass resolution. High purity nitrogen was used for both the source and collision gas flows. In the first quadrupole single charged ions $[M + H]^+$ were selected. All precursor ions, product ions, optimum fragmentor voltage and collision energy values are shown in table 1.

The most intense daughter ion in the product spectrum was chosen as quantifier, and a second daughter ion was chosen as qualifier. However, for methylphenidate, the fragmentation of the ionized molecule only resulted in one daughter ion in the mass spectrum, so only one mass transition (quantifier) for methylphenidate was used. Peak height ratios of the substance and its internal standard were used to calculate concentrations. For all substances the capillary voltage was set at 4,000 V, gas temperature at 320°C, gas flow at 13 L/min, nebulizer gas at 60 psi, sheath gas temperature at 400°C, sheath gas flow at 12 L/min and the nozzle voltage at 0 V. The Agilent 1200 series autosampler was set at 10°C and the integrated column oven was set at a temperature of 40°C. Analyses were performed on a 50 x 2.1 mm Hypurity Aquastar, 5-µm analytical column from ThermoFisher Scientific (Waltham, MA, USA) equipped with a separate 0.5 µm Varian frit filter (Palo Alto, USA). Chromatographic

Table 1 Mass spectrometer settings for all substances

Substance	Precursor ion (m/z)	Product ion Quantifier (m/z)	Product ion Qualifier (m/z)	Fragmentor voltage (V)	CE Quantifier (V)	CE Qualifier (V)
Amphetamine	136.3	91.2	119.2	70	8	3
Amphetamine-D_{10}	146.2	98.2	n.a.	70	8	n.a.
Methamphetamine	150.2	91.1	119.2	80	12	5
Methamphetamine-D_5	155.3	91.1	n.a.	80	12	n.a.
MDMA	194.2	105.2	133.2	80	23	15
MDMA-D_3	197.3	105.1	n.a.	80	23	n.a.
MDA	180.2	105.2	133.2	70	20	15
MDA-D_5	185.3	110.2	n.a.	70	20	n.a.
MDEA	208.2	105.2	133.2	95	25	18
MDEA-D_5	213.4	105.1	n.a.	95	25	n.a.
Methylphenidate	234.2	84.2	n.a.	90	16	n.a.
Methylphenidate-D_9	243.4	93.2	n.a.	90	16	n.a.
Cocaine	304.2	182.2	150.2	130	15	23
Cocaine-D_3	307.3	185.2	n.a.	130	15	n.a.
Benzoylecgonine	290.2	168.2	105.1	120	15	30
Benzoylecgonine-D_3	293.2	171.2	n.a.	120	15	n.a.
Morfine	286.2	165.2	153.2	160	45	50
Morfine-D_3	289.3	165.2	n.a.	160	45	n.a.
Codeine	300.2	165.2	153.1	155	45	50
Codeine-D_3	303.3	165.2	n.a.	155	45	n.a.
6-MAM	328.2	165.3	211.2	120	45	20
6-MAM-D_3	331.2	165.2	n.a.	120	45	n.a.
Heroin	370.2	165.2	268.2	170	55	25
Heroin-D_3	373.4	165	n.a.	170	55	n.a.
Methadone	310.3	265.2	105.1	110	10	25
Methadone-D_3	313.3	268.2	n.a.	110	10	n.a.
EDDP	278.3	234.2	186.2	160	30	35
EDDP-D_3	281.3	234.2	n.a.	160	30	n.a.
Nicotine	163.2	130.1	106.2	100	16	10
Nicotine-D_4	167.3	134.1	n.a.	100	16	n.a.
Cotinine	177.2	80.2	98.1	140	20	14
Cotinine-D_3	180.2	80.2	n.a.	140	20	n.a.

CE is collision energy.

separation was performed by means of a gradient with a flow of 0.5 mL/min and a run time of 9.6 minutes. The gradient started at 100% 20 mmol/L ammonium formate buffer pH 7.0 with 5% v/v methanol and increased to 100% methanol in 6 minutes to maintain at 100% methanol from 6 to 7.8 minutes. At 7.81 minutes the gradient returned to 100% 20 mmol/L ammonium formate buffer pH 7.0 with 5% methanol and stabilized until 9.6 minutes in order to prepare the chromatographic system for the next injection. Agilent Masshunter software for quantitative analysis (version B.04.00) was used to quantify the analysis results.

Preparation of stock solutions and extraction solvent

Reference solutions of 1,000 mg/L for each substance were used to prepare two stock solutions in methanol containing 50 mg/L nicotine and 5 mg/L for all other substances. One set of reference solutions was used to prepare the stock solution used for the calibration curve and a separate set was used to prepare the stock solution for the quality control samples. A combined stock solution of 5 mg/L was also prepared for all the deuterated IS. This combined IS stock solution was used to spike the extraction solvent at 25 µg/L: the latter consisted of analytical grade 20 mmol/L ammonium formate buffer pH7:methanol (50:50 % v/v).

Sample preparation

For the extraction of the analytes the patches (PharmChem, Inc., Fort Worth, Texas, USA) were transferred to polypropylene containers (Kartell; Noviglio, Italy) using tweezers and 1.5 mL extraction solvent was added. The samples were extracted by vigorous shaking at 1,800 rpm for 10 minutes using a Retsch MM400 mixer mill (Haan, Germany). Of the extract 200 µL was transferred into a Whatman mini-Uniprep™ syringeless filter vial (Maidstone, England) and 15 µL of the filtered supernatant was injected into the LC-MS/MS system.

Method validation

For validation, extracts of blank sweat patches were collected for spiking the calibration and validation samples. The calibration curve for all substances other than nicotine were spiked at 3.0, 6.0, 15, 38, 75, 150, 300, 375 ng/patch. The accuracy and precision concentrations were spiked at the Lower Limit Of Quantification (LLOQ 1 and 2), Low, Medium (Med) and High. The validation concentrations for all substances other than nicotine were spiked at 3.0, 6.0, 25, 150, and 300 ng/patch. The calibration curve for nicotine was spiked at 30, 60, 150,

375, 750, 1,500, 3,000 and 3,750 ng/patch. The accuracy and precision concentrations for nicotine were spiked at 30, 60, 250, 1,500, and 3,000 ng/patch. Validation was performed with a maximum tolerated bias and CV of 20% for the LLOQ and 15% for the other validation concentrations, including the stability validation. For accuracy and precision all concentrations were measured five-fold in three separate runs on three separate days. For each accuracy and precision concentration bias and CV were calculated per run. Within-run, between-run and overall CVs were calculated with the use of one-way ANOVA. Eight calibration points were used to determine linearity on three separate days. Stability of the substances was assessed in worn patches at room temperature and in processed samples in the auto-sampler at 10°C at Low level. The patches used for stability testing were worn for one week by five non-drug using volunteers, where each volunteer wore 2 patches on their upper arm. After one week of wearing, one patch from each volunteer was spiked with all substances at 75 ng/patch for all substances except for nicotine, which was spiked at 750 ng/patch. These spiked worn patches were left at ambient temperature for one week. At the end of that week the other worn patch of each volunteer was spiked at the same levels to be measured as time zero. The patches spiked at time zero were also re-injected to determine the autosampler stability for 5 days. For both stability tests CV and bias should have been less than 15%. Selectivity and specificity was assessed by analyzing 6 blank sweat patches worn by 6 non-drug using volunteers. Carry-over was monitored during validation, where the peak height of the first blank following the highest calibrator should not exceed 20% of the peak height at the LLOQ. During the method validation the ion ratios for the qualifier and quantifier were evaluated at the LLOQ 1, LLOQ 2 and Low levels of the accuracy and precision samples. The ratio between the qualifier and the quantifier was required to be within 20% of the ratio set in each validation run. Analytical cut-off concentrations were defined for each substance at the lowest validated accuracy and precision concentration with a bias and CV within 15% and all qualifier/quantifier ratios within 20% of the set ratio during the whole validation. Matrix effects were investigated for extracts of un-worn and worn sweat patches. To investigate these effects five sweat patches were worn for one week by different non-drug using volunteers. After extraction of the worn and un-worn sweat patches the extracts and blank extraction solvents were spiked at 10 and 50 ng/patch for all substances other than nicotine, which was spiked at 100 and 500 ng/patch. The matrix effects were calculated as follows. Matrix effect caused by the patch: (100 x mean peak height of spiked extracts of a blank un-worn patch / mean peak height of spiked neat solution) - 100. Matrix effect caused by sweat: (100 x mean peak height of spiked extracts of a blank worn patch / mean peak height of spiked extracts of a blank un-worn patch) - 100.

Routine analysis

For routine analysis sweat patches were collected from patients with a mild or borderline intellectual disability. These patients were under institutional care for their disability. The analyzed sweat patches were used to validate a questionnaire for drug abuse in this target group [1, 2]. All patients (and if applicable, their guardians) gave informed consent to participate in the study, which was approved by a certified medical ethical committee. For patient monitoring the validated analytical cut-off concentrations were used to determine drug abuse instead of those proposed by the SAMHSA guidelines.

RESULTS

Method validation

The validation results regarding linearity, accuracy and precision and stability are shown in table 2. All substances other than nicotine were validated with a linear range of 3.0 to 375 ng/patch. Nicotine was validated with a linear range of 30 to 3,750 ng/patch. Nicotine had a large bias of 22.3% at LLOQ 1 (30 ng/patch). Therefore the validated LLOQ of nicotine was set at LLOQ 2 (60 ng/patch), with a bias and CV of -1.6% and 3.5%, respectively. For the other substances the highest overall bias found during the accuracy and precision validation was -14.9% for LLOQ 1 of methylphenidate while the highest overall CV was 9.2% for LLOQ 1 of amphetamine. The auto-sampler stability in extracts of worn sweat patches was proven for 5 days for all substances, with a maximum overall bias of 14.7% for amphetamine. Stability of all substances in worn sweat patches were assessed at ambient temperature for 7 days and showed that heroin was unstable, with a bias and CV of 22.1% and -30.6%, respectively. The high CV showed that the instability of heroin was influenced by patient matrix variability and varied from -50.4% to -13% bias. For all other substances the stability test at ambient temperature showed the highest bias of -8.8% for cocaine and the highest CV of 13.3% for 6-MAM.

The mean ion ratios of the qualifier and quantifier and their maximum deviations found during the validation are shown in table 3. It can be observed that already at LLOQ 1 level all substances were below -13.8% bias of the set ion-ratio: at LLOQ 2 and Low the highest deviations were -12.9% and -7.2%, respectively.

The combined results of the accuracy and precision and monitored ion ratios showed validated analytical cut-off concentrations of 3.0 ng/patch for all substances but nicotine,

Table 2 Validation results for the linearity, accuracy, precision, autosampler stability at 5 days (AS) and stability at ambient temperature in spiked worn patches for 7 days (RT)

Substance	Correlation coefficient linear range (pg/mg)	Concentration (ng/patch)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall bias (%)
Amphetamine	0.9963	LLQ 1 (3.0)	1.5	9.1	9.2	-13.9
	3.0–375	LLQ 2 (6.0)	1.6	1.6	2.3	-7.2
		LOW (25)	1.3	5.3	5.4	1.3
		MED (150)	2.1	3.1	3.7	-1.4
		HIGH (300)	1.3	0.0	1.3	-3.3
		AS stab (75)	n.a.	n.a.	6.0	14.7
		RT stab (75)	n.a.	n.a.	6.1	-8.5
Methamphetamine	0.9995	LLQ 1 (3.0)	1.6	1.9	2.5	1.4
	3.0–375	LLQ 2 (6.0)	1.3	1.3	1.8	-1.3
		LOW (25)	1.0	0.5	1.1	-1.3
		MED (150)	1.1	0.6	1.2	0.8
		HIGH (300)	1.3	0.0	1.3	4.7
		AS stab (75)	n.a.	n.a.	1.1	1.4
		RT stab (75)	n.a.	n.a.	5.1	-1.8
MDMA	0.9996	LLQ 1 (3.0)	4.9	2.1	5.3	-7.1
	3.0–375	LLQ 2 (6.0)	2.3	3.0	3.7	-6.5
		LOW (25)	1.8	3.7	4.1	2.7
		MED (150)	1.0	4.6	4.7	-0.1
		HIGH (300)	1.1	3.7	3.9	0.7
		AS stab (75)	n.a.	n.a.	1.0	2.5
		RT stab (75)	n.a.	n.a.	6.8	-3.0

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Table 2 ***Continued***

Substance	Correlation coefficient linear range (pg/mg)	Concentration (ng/patch)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall bias (%)
MDA	0.9991	LLQ 1 (3.0)	3.0	0.0	3.0	3.3
	3.0–375	LLQ 2 (6.0)	1.3	2.8	3.1	-3.9
		LOW (25)	1.4	2.5	2.9	-2.1
		MED (150)	1.3	3.6	3.8	-2.0
		HIGH (300)	1.2	2.6	2.8	1.9
		AS stab (75)	n.a.	n.a.	2.4	0.8
		RT stab (75)	n.a.	n.a.	4.2	-6.3
MDEA	0.9992	LLQ 1 (3.0)	3.8	2.1	4.4	-11.8
	3.0–375	LLQ 2 (6.0)	2.5	1.5	2.9	-9.5
		LOW (25)	0.8	3.4	3.5	-2.2
		MED (150)	1.4	4.7	4.9	-3.5
		HIGH (300)	0.8	4.2	4.3	-2.3
		AS stab (75)	n.a.	n.a.	1.2	3.3
		RT stab (75)	n.a.	n.a.	4.6	-3.3
Methylphenidate	0.9991	LLQ 1 (3.0)	3.2	6.5	7.2	-14.9
	3.0–375	LLQ 2 (6.0)	1.7	1.7	2.4	-8.1
		LOW (25)	1.6	4.7	4.9	2.3
		MED (150)	1.1	4.9	5.0	0.4
		HIGH (300)	1.2	2.9	3.1	-1.7
		AS stab (75)	n.a.	n.a.	5.7	8.5
		RT stab (75)	n.a.	n.a.	4.9	-3.8

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Table 2 ***Continued***

Substance	Correlation coefficient linear range (pg/mg)	Concentration (ng/patch)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall bias (%)
Cocaine	0.9994	LLQ 1 (3.0)	1.5	0.6	1.6	-8.0
	3.0–375	LLQ 2 (6.0)	1.2	3.1	3.3	-9.2
		LOW (25)	0.8	0.6	1.0	-5.5
		MED (150)	0.4	0.8	0.9	-3.7
		HIGH (300)	0.9	0.0	0.9	0.0
		AS stab (75)	n.a.	n.a.	1.0	-3.1
		RT stab (75)	n.a.	n.a.	4.8	-8.8
Benzoylecgonine	0.9995	LLQ 1 (3.0)	1.3	1.2	1.8	2.1
	3.0–375	LLQ 2 (6.0)	1.2	1.6	2.0	-3.1
		LOW (25)	0.6	0.7	1.0	-0.8
		MED (150)	0.4	0.6	0.4	-1.3
		HIGH (300)	0.6	1.0	1.1	2.2
		AS stab (75)	n.a.	n.a.	1.1	1.8
		RT stab (75)	n.a.	n.a.	8.8	-0.5
Morphine	0.9991	LLQ 1 (3.0)	4.2	2.0	4.6	-1.6
	3.0–375	LLQ 2 (6.0)	2.4	2.9	3.8	-5.8
		LOW (25)	1.6	0.4	1.6	-1.4
		MED (150)	1.6	1.1	1.6	-1.4
		HIGH (300)	1.0	0.6	1.2	0.8
		AS stab (75)	n.a.	n.a.	3.2	3.6
		RT stab (75)	n.a.	n.a.	5.9	-4.3

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Table 2 ***Continued***

Substance	Correlation coefficient linear range (pg/mg)	Concentration (ng/patch)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall bias (%)
Codeine	0.9993	LLQ 1 (3.0)	4.4	3.7	5.7	-8.8
	3.0–375	LLQ 2 (6.0)	4.3	1.0	4.4	-7.3
		LOW (25)	1.9	0.8	2.1	-0.4
		MED (150)	2.2	0.9	2.4	-0.5
		HIGH (300)	2.0	0.8	2.1	-0.1
		AS stab (75)	n.a.	n.a.	2.3	1.4
		RT stab (75)	n.a.	n.a.	6.7	-3.2
6-MAM	0.9982	LLQ 1 (3.0)	2.8	4.1	5.0	0.4
	3.0-375	LLQ 2 (6.0)	1.9	0.0	1.9	-3.1
		LOW (25)	4.7	0.0	4.7	0.3
		MED (150)	7.1	0.0	7.1	-0.4
		HIGH (300)	1.3	6.5	6.6	3.2
		AS stab (75)	n.a.	n.a.	5.1	-4.5
		RT stab (75)	n.a.	n.a.	13.3	0.1
Heroin	0.9962	LLQ 1 (3.0)	5.3	1.6	5.5	-7.0
	3.0–375	LLQ 2 (6.0)	3.4	6.0	6.9	-13.2
		LOW (25)	3.3	3.0	4.4	-8.0
		MED (150)	2.9	2.8	4.0	-6.5
		HIGH (300)	3.6	4.0	5.4	-6.6
		AS stab (75)	n.a.	n.a.	3.6	2.7
		RT stab (75)	n.a.	n.a.	22.1	-30.6

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Table 2 ***Continued***

Substance	Correlation coefficient linear range (pg/mg)	Concentration (ng/patch)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall bias (%)
Methadone	0.9993	LLQ 1 (3.0)	0.9	2.9	3.1	2.4
	3.0–375	LLQ 2 (6.0)	0.7	1.0	1.2	-2.4
		LOW (25)	0.7	2.0	2.1	-1.1
		MED (150)	0.6	2.0	2.1	-0.2
		HIGH (300)	1.0	2.7	2.9	4.2
		AS stab (75)	n.a.	n.a.	0.8	2.2
		RT stab (75)	n.a.	n.a.	4.4	-4.1
EDDP	0.9993	LLQ 1 (3.0)	0.7	1.3	1.5	8.0
	3.0–375	LLQ 2 (6.0)	1.0	0.0	1.0	-3.2
		LOW (25)	0.7	0.5	0.9	-7.7
		MED (150)	0.6	0.0	0.6	-1.0
		HIGH (300)	0.6	0.8	1.0	5.9
		AS stab (75)	n.a.	n.a.	0.8	4.3
		RT stab (75)	n.a.	n.a.	3.2	-4.4
Nicotine	0.9985	LLQ 1 (30)	2.6	6.4	7.0	22.3
	30–3,750	LLQ 2 (60)	2.7	2.3	3.5	-1.6
		LOW (250)	2.2	4.5	5.0	-3.6
		MED (1500)	3.4	0.0	3.4	-4.0
		HIGH (3000)	3.4	1.8	3.8	-1.5
		AS stab (750)	n.a.	n.a.	2.8	0.6
		RT stab (750)	n.a.	n.a.	8.2	-0.1

Table 2 continues on next page

Table 2 ***Continued***

Substance	Correlation coefficient linear range (pg/mg)	Concentration (ng/patch)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall bias (%)
Cotinine	0.9993	LLQ 1 (3.0)	1.0	0.5	1.1	3.1
	3.0–375	LLQ 2 (6.0)	0.9	1.3	1.6	-4.1
		LOW (25)	0.6	0.0	0.6	-3.1
		MED (150)	0.4	0.5	0.6	-4.2
		HIGH (300)	0.5	0.6	0.8	-1.0
		AS stab (75)	n.a.	n.a.	1.1	5.9
		RT stab (75)	n.a.	n.a.	4.2	2.5

For the accuracy and precision, all concentrations were measured five-fold in three separate runs on three separate days. For linearity, a single calibration curve was measured on 3 separate days.

Table 3 Mean qualifier/ quantifier ratios during the validation and the maximum deviations found at LLOQ 1, LLOQ 2 and Low levels

		Maximum deviation %		
Substance	Mean ion-ratio	LLOQ 1 3 ng/patch	LLOQ 2 6 ng/patch	Low 25 ng/patch
Amphetamine	70	6.4	-2.6	-1.3
Methamphetamine	30	6.1	5.8	-3.4
MDMA	63	-11.2	10.5	3.9
MDA	69	9.1	5.2	-2.4
MDEA	63	-9.1	7.4	3.5
Methylphenidate	n.a.	n.a.	n.a.	n.a.
Cocaine	8.0	-11.9	7.1	-7.1
Benzoylecgonine	29	4.9	5.2	-2.2
Morphine	86	-8.4	-12.9	-2.6
Codeine	81	-13.8	-10.5	-7.2
6-MAM	64	10.3	-11.5	4.1
Heroin	67	15.4	12.1	4.4
Methadone	48	-3.6	-3.9	-2.5
EDDP	30	-5.4	-3.8	2.1
Nicotine	46	6.9	-7.5	-5.6
Cotinine	23	-4.7	6.9	2.3

All concentrations were measured five-fold in three separate runs on three separate days.

which was 60 ng/patch. The cut-off concentrations based upon the analytical performance and the cut-off concentrations set by the SAMHSA guidelines are shown in table 4.

All validated analytical cut-off concentrations were well below the cut-off concentrations set by the SAMHSA, where provided. In figure 1, the combined chromatograms of all validated substances at their validated analytical cut-off concentrations are shown.

Selectivity and specificity showed no interfering peaks of more than 20% of the LLOQ. No carry-over was detected during method development and validation. During method development the extraction recoveries of the substances from the patch were investigated and showed that the extraction efficiencies ranged from 93% to 100% (data not shown). In table 5 the matrix effects are shown for worn and un-worn patches. The results show that the patch itself already causes matrix effects ranging from -42% for heroin to +2% for EDDP. Matrix effects caused by sweat ranged from -23% for amphetamine to +28% for heroin. Since

Table 4 Validated analytical cut-off concentrations based on the performance of the quantifier and qualifier and the cut-off concentrations set by the SAMHSA

Substance	Analytical Cut-off ng/patch	SAMHSA Cut-off ng/patch
Amphetamine	3.0	25
Methamphetamine	3.0	25
MDMA	3.0	25
MDA	3.0	25
MDEA	3.0	25
Methylphenidate	3.0	n.a.
Cocaine	3.0	25
Benzoylecgonine	3.0	25
Morphine	3.0	25
Codeine	3.0	25
6-MAM	3.0	25
Heroin	3.0	n.a.
Methadone	3.0	n.a.
EDDP	3.0	n.a.
Nicotine	60	n.a.
Cotinine	3.0	n.a.

all validation concentrations were spiked in extracts of blank un-worn patches, these matrix effects are accounted for. It appeared that the response of heroin was increased by matrix effects of the worn sweat patch. For each substance, matrix effects were compensated for by their deuterated internal standard.

Routine analysis

The analytical method was used for the analysis of 96 sweat patches from 78 adult subjects. Each patch was worn for 7 consecutive days. Some subjects wore multiple patches, which were worn consecutively. During the routine analysis methylphenidate, cocaine, codeine, nicotine and cotinine were detected above the validated analytical cut-off concentrations in the analyzed sweat patches. Acceptable ratios of the qualifier/quantifier mass transitions were found for each positive sample. 69 patches tested positive (>60 ng/patch) for the presence of nicotine, of which 57 were confirmed by the presence of cotinine (>3.0 ng/

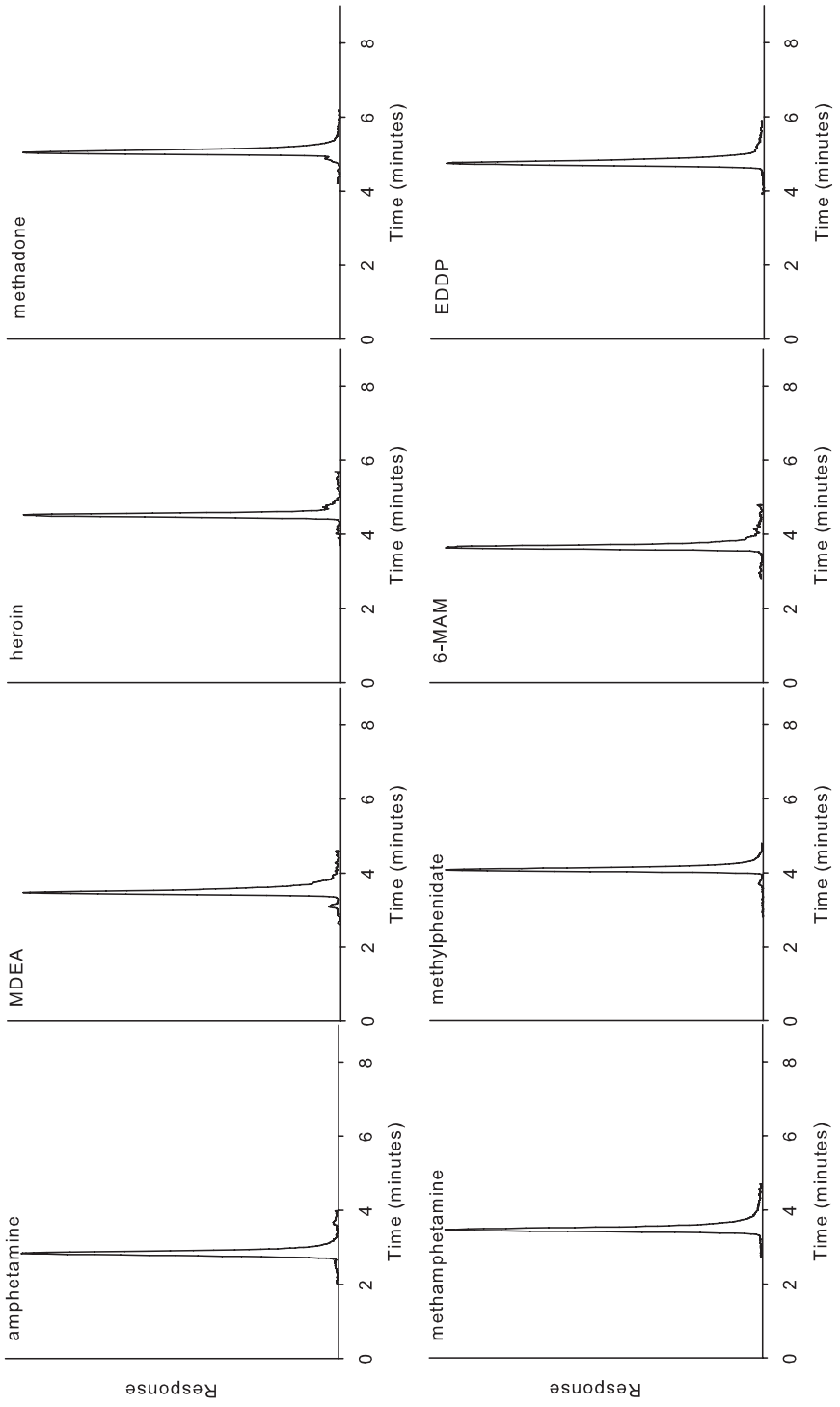

Figure 1 Chromatograms of the validated analytical cut-off concentrations.
The chromatograms represent the following concentrations: 60 ng/patch for nicotine and 3.0 ng/patch for all other validated substances.

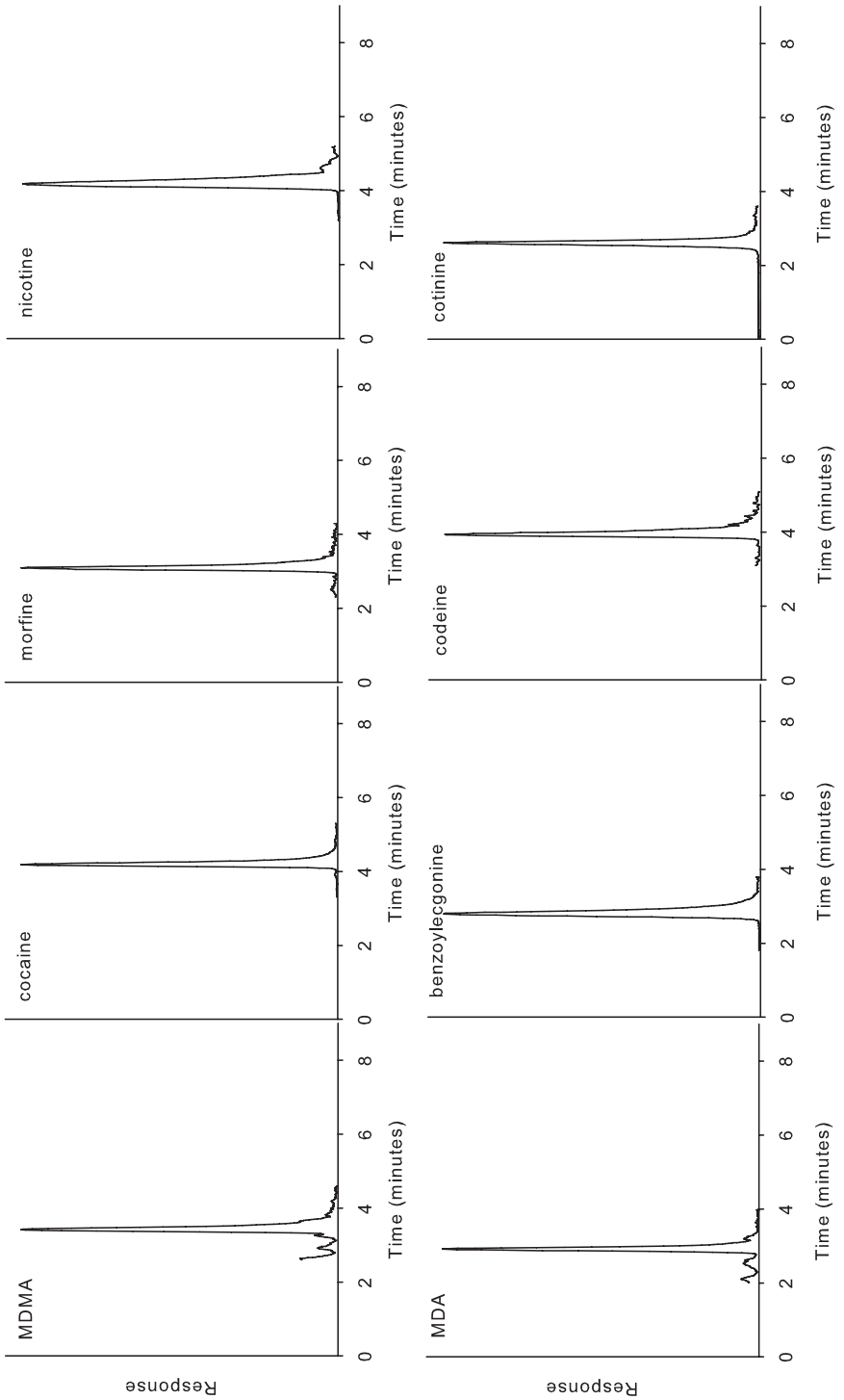

Figure 1 ***Continued.***

Table 5 Matrix effects of blank un-worn patches and blank worn patches

Substance	Matrix effect caused by the patch %	Matrix effect caused by sweat %
Amphetamine	-26	-23
Methamphetamine	-17	-6
MDA	-35	-13
MDEA	-27	-2
MDMA	-31	-1
Methylphenidate	-4	-7
Cocaine	-3	-11
Benzoylecgonine	-10	-13
Morphine	-24	-9
Codeine	-11	-15
6-MAM	-18	-4
Heroin	-42	+28
Methadone	-1	-12
EDDP	2	-7
Nicotine	1	-16
Cotinine	-23	-16

patch). The concentrations found for nicotine ranged from 60 to 17,224 ng/patch and for cotinine from 4.0 to 570 ng/patch. Methylphenidate was present in 15 of the analyzed sweat patches, with concentrations ranging from 4.7 to 76 ng/patch. This led to the conclusion that the subjects had used methylphenidate, either as a prescription drug or as drug of abuse. One subject had 8.6 ng cocaine present in the sweat patch, but no benzoylecgonine was detected. Without the presence of benzoylecgonine the use of cocaine cannot be confirmed. Codeine was present in 4 sweat patches, with concentrations ranging from 4.9 to 67 ng/patch.

DISCUSSION

This LC-MS/MS method has been developed for fast and highly selective screening of drugs present in PharmCheck™ sweat patches used by patients who have been monitored for drug abuse. The fast extraction and analysis of the sweat patches provides a short turn-around time and the method was fully validated with enhanced selectivity by validated qualifier/ quantifier ratios. The identification criteria concerning the performance of the qualifier

and quantifier ratios set by the Commission of the European Communities was considered not strict enough for the validation of the method described here [24]. The qualifier mass transition of cocaine for example has an ion ratio of only 8% compared to its quantifier. According to the identification criteria set by the Commission of the European Communities this means an extremely large maximum permitted tolerance of ±50%. To ensure the selectivity and reliability of our method the maximum deviation from the standardized ratio was set at 20% for all substances. Despite the more strict requirements for the qualifier and quantifier ratios, the validation yielded low analytical cut-off concentrations with validated qualifier/quantifier ratios. All validated analytical cut-off concentrations of 3 ng/patch were well below the cut-off concentrations of 25 ng/patch set by the SAMHSA guidelines, where provided. Previously published methods describe the determination of a single substance or a group of similar substances, such as nicotine [26], methadone [17], cocaine [16, 27] or opiates [28, 29]. A number of GC-MS publications describe the simultaneous analysis of up to 10 multiple substances, using time consuming extraction procedures [13, 22, 30]. Only one article has described sweat patch analysis of multiple substances performed with LC-MS/MS, but that method still used an extensive sample preparation with SPE [23].

In the method described by Concheiro et al. 14 drugs were analyzed in sweat patches using SPE and LC-MS/MS with a runtime of 15 minutes and the use of qualifier mass transitions [23].

Despite the application of SPE to clean up the sample, ion-suppression and enhancement was still observed for a couple of substances, while the extraction efficiency of the SPE procedure only yielded about 50%. The application of an extensive SPE method can decrease a significant part of the ion-suppression caused by the sample matrix. However, a multi-analyte SPE method should be selective for all analyzed substances. This makes the SPE less selective in total and thus less capable of separating interfering substances that may cause ion-suppression. In addition, most ion-suppression is observed in the front of the chromatogram and should be well separated from the analyte peaks through the chromatographic gradient, regardless of the sample preparation. The time and effort invested in extensive and time consuming sample preparations hardly seems justified, as they are not able to fully eliminate ion-suppression effects and recoveries are not always high. The use of LC-MS/MS and deuterated internal standards (IS) provides low LLOQs without the need to perform extensive sample preparation to concentrate the sample. The use of qualifier mass transitions ensures that the selectivity of GC-MS can be matched with the use of LC-MS/MS. Compared to previously published methods, our method uses a fast and simple extraction and shorter LC-MS/MS runtimes, providing short turnaround times for sweat patch screening.

A previously described stability validation was performed in spiked patches at ambient temperature and found no instability of the investigated substances [23]. In our study the stability validation was performed in worn sweat patches and showed degradation of heroin, with high individual variability. In addition, a somewhat higher CV of 13.3% was also present for the stability testing of 6-MAM. The individual patches that showed the most heroin instability also showed slightly increased 6-MAM concentrations. This indicates that individual matrix variability can influence degradation of heroin to 6-MAM even when the patch is removed and stored at ambient temperature. Therefore, positive results for 6-MAM should be interpreted with caution when the patch is stored at ambient temperature prior to analysis.

Our method was used to analyse 96 sweat patches worn by 78 subjects. The concentrations found for nicotine ranged from 60 to 17,224 ng/patch and for cotinine from 4.0 to 570 ng/patch. Kintz et al. monitored for nicotine in sweat patches after 3 days' wear and found concentrations ranging from 150 to 2,498 ng/patch [26]. Concheiro et al. found a maximum concentration of 202 ng/patch cotinine in a pregnant subject [23]. In the studies of Marchei et al. methylphenidate was administered to subjects and multiple patches were applied to each subject [19, 31]. At different time points, patches were removed for analysis. Maximum concentrations of methylphenidate found in the study of Marchei et al. reached 34 ng/patch when a patch was worn for 25 hours. The concentrations of methylphenidate found in our subjects ranged from 4.7 to 76 ng/patch. The higher concentrations found in our sweat patches for nicotine, cotinine and methylphenidate are most probably due to the extended length of patch wear of one week which may have accumulated multiple doses of the ingested substance.

Codeine was present in 4 sweat patches at concentrations ranging from 4.9 to 67 ng/patch. Kintz et al. found 73 ng/patch for codeine in an addicted subject using street heroine contaminated with codeine [32]. Brunet et al. found a maximum of 196 ng/patch codeine in sweat patches from pregnant women in a methadone maintenance treatment program [12]. Although in our study the detected codeine could have originated from contaminated street heroin, no heroin or 6-MAM were detected. The detected codeine had probably been used as an analgesic and to relieve cough.

In the study of Taylor et al. sweat patch analysis was compared to urine analysis in a methadone clinic and good agreement was found for most drugs of abuse tested [33]. Weekly urine analysis may have even been the main reason for possible deterioration of the agreement,

since cocaine is excreted from the body within 3 days and single use of cocaine may not be detected, while the sweat patch was able to accumulate drugs during the whole week. Most screening for drugs of abuse is performed in urine. With cocaine excretion within 3 days after use, at least 2 urine samples are needed per week in order to monitor drug abuse. The easy and fast sweat patch extraction combined with the high LC-MS/MS throughput makes weekly sweat patch screening with LC-MS/MS a good alternative to urine testing. Combined with the high sensitivity and selectivity of our method, sweat patch analysis proved to be a very useful complementary tool for physicians and health care professionals to evaluate possible drug abuse in their specific patient setting.

CONCLUSION

The method described here uses a simple extraction without extensive sample treatment for the analysis of 16 drugs and metabolites with a runtime of 9.6 minutes and was extensively validated regarding cut-off concentrations, qualifier/quantifier ratios and stability assessment in worn sweat patches. The validation resulted in much lower analytical cut-off concentrations than those set by the SAMHSA guidelines, while using more strict requirements for qualifier confirmation than proposed by the European Commission [10, 24]. This method was successfully applied to the analysis of 96 worn sweat patches to monitor patients for drug abuse. The use of sweat patches provides an alternative matrix to urine or blood samples and patients can be monitored for drug abuse without invasive sample collection. The sweat patch analysis provided the physicians with information about drug abuse, which can be used for patient specific therapy.

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