Efficiently Automated Drug Analysis

Comprehensive automation of SPE-GC/MS based analysis of serum and other matrices for opioids, cocaine and metabolites.

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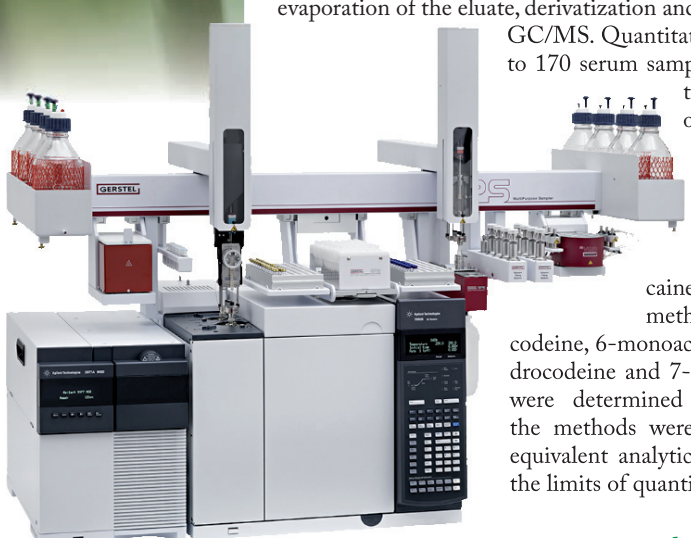
Analyzing blood serum for opioids, cocaine and metabolites is a routine task in forensic laboratories. The most commonly used methods involve several manual or partly-automated sample preparation steps such as protein precipitation, solid phase extraction, evaporation and derivatization followed by GC/MS or LC/MS determination. In the work reported here, a comprehensively automated method is compared with a validated, partly-automated routine method. Following manual protein precipitation, the automated method relies on a GERSTEL MultiPurpose Sampler (MPS) to perform all remaining sample preparation steps. These include solid phase extraction (SPE), evaporation of the eluate, derivatization and introduction to the GC/MS. Quantitative analysis of close to 170 serum samples, as well as more than 50 samples of other matrices like urine, different tissues and heart blood, was performed using both methods. Cocaine, benzoylecgonine, methadone, morphine, codeine, 6-monoacetylmorphine, dihydrocodeine and 7-aminoflunitrazepam were determined quantitatively and the methods were found to produce equivalent analytical results even near the limits of quantification.



GC/MS System used for the determination of opioids, cocaine, and metabolites in serum, as well as for THC and metabolites in serum [3]. The system performs automated SPE, evaporative concentration, derivatization as well as introduction to the GC/MS.

Instrumentation

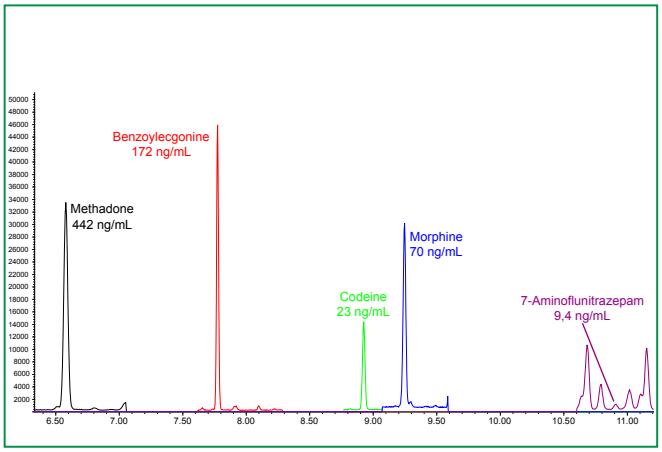
A GERSTEL MultiPurpose Sampler (MPS) was used, configured with a 2.5 mL syringe with gas supply for the sample preparation steps and a 10

Source

[1] O. Lerch, O. Temme, T. Daldrup: „Comprehensive automation of the solid phase extraction gaschromatographic mass spectrometric analysis (SPE-GC/MS) of opioids, cocaine, and metabolites from serum and other matrices“, Anal. Bioanal. Chem. 406 (2014) 4443; Free download of the article under: http://link.springer.com/article/10.1007/s00216-014-7815-7

[2] O. Lerch, O. Temme, T. Daldrup: „Comprehensive Automation of the SPE-GC/MS Analysis of Opioids, Cocaine and Metabolites from Serum and Other Matrices“, GERSTEL AppNote 07/2013 (www.gerstel.com/pdf/p-gc-an-2013-07.pdf)

Standard solid phase extraction cartridge (bottom) adapted for automated SPE.



Extracted ion chromatogram resulting from a real serum sample. Quantified compounds are named.

µL syringe used for sample injection into a GERSTEL Cooled Injection System (CIS 4) coupled to a 7890 GC/5975 MSD (Agilent Technologies). The MPS was equipped with modules for solid phase extraction (GERSTEL SPE), for evaporation of solvents under controlled vacuum and temperature (GERSTEL ™VAP), for shaking under controlled temperature conditions (Agitator) and for supplying large volumes of solvents (GERSTEL SFS).

Materials

All analytes and deuterated analogues were certified standards purchased from Lipomed AG or LGC Promochem GmbH. All solvents and salts were of analytical grade and purchased from VWR. N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) for silylation was purchased from Macherey-Nagel or Sigma-Aldrich. Bond Elut Certify 130 mg, 3 mL format SPE cartridges from Agilent Technologies were used. For automated SPE these cartridges were cut at the top, equipped with a transport adapter and a disposable syringe needle (canula). Blood, urine and tissue samples were taken from authentic forensic cases at the Institute of Legal Medicine in Düsseldorf.

Preparation of standards and solutions

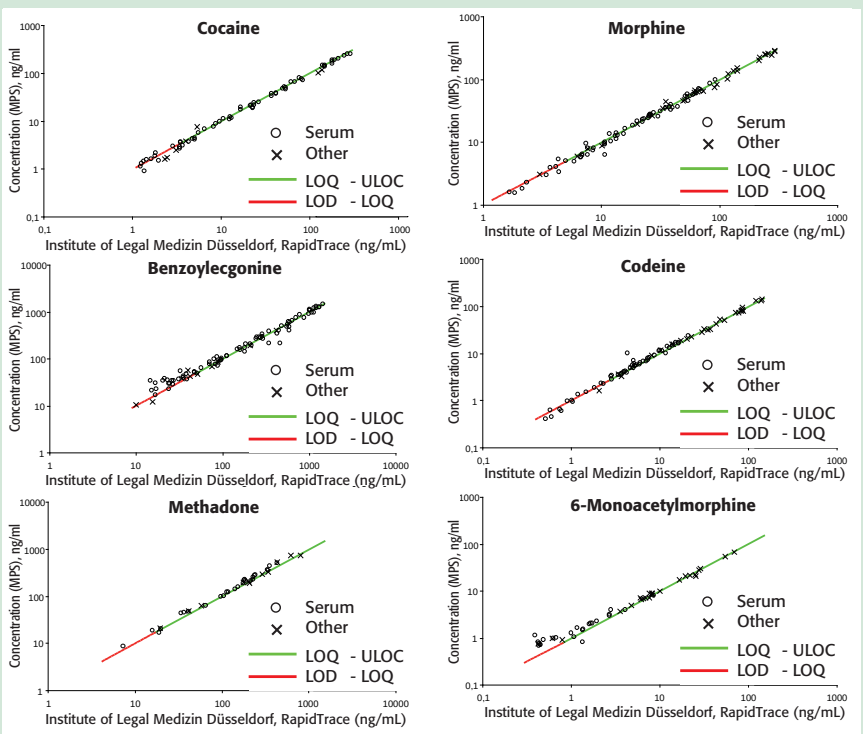
For calibration, multi-compound calibration solutions and one multi-compound internal standard solution containing deuterated analogues of every analyte were prepared in methanol. The calibrations ranged from 25 to 1500 ng/mL (methadone), from 50 to 1500 ng/mL (benzoylecgonine), from 5 to 150 ng/mL (codeine), from 5 to 300 ng/mL (cocaine, dihydrocodeine, morphine), and from 2.5 to 150 ng/mL (7-aminoflunitrazepam, 6-monoacetylmorphine) respectively and were calculated for 0.6 mL serum sample (nine levels). A 20 µL aliquot of the internal standard solution was added to each individual sample, calibration sample and quality control sample.

In agreement with the guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh, Germany) a blank injection of pure derivatization solution was performed after every sample, quality control or calibration sample.

Results and Discussion

The validated, partly-automated routine analysis method was successfully automated using the MPS – from dilution of the sample after protein precipitation to injection into the GC/MS. Some modifications were necessary:

Dilution of the supernatant after protein precipitation was partly performed in the autosampler syringe. A 0.75 mL aliquot of the supernatant was diluted with 0.75 mL phosphate buffer and 0.75 mL of this mixture was aspirated. After that, another 1.75



Correlation of determined analyte concentrations in double logarithmic scale. Line with a slope of one – representing complete equivalence of results – is shown.

ng/mL: Nanogram per milliliter or nanogram per gram for tissue respectively; Other: Other matrices than serum - urine, blood, lyophilized kidney tissue, heart blood, lyophilized and native brain tissue; LOD: Limit of detection; LOQ: Limit of quantification; ULOC: Upper limit of calibration.

Analysis Conditions

MPS

Syringe:	10 µL
Injection Volume:	2 µL

CIS inlet

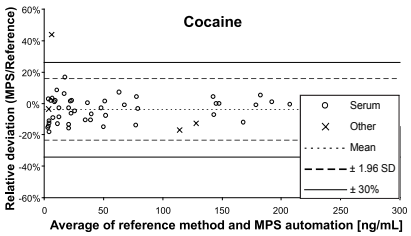
Temperature:	50 °C -12 °C/s - 280 °C (5 min)
Pneumatics:	Splitless 3 min
Liner:	Quartz wool deactivated

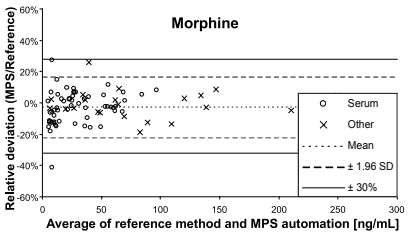
GC

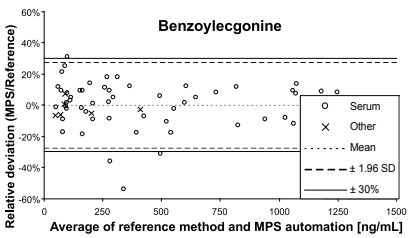
Oven Temperature:	140 °C (1 min) - 120 °C/min - 225 °C (5.29 min) - 120 °C/min - 275 °C (5.2 min)
Post Run:	300 °C (2.5 min)
Column:	Rxi-5Sil MS (Restek) 30 m, di = 0.25 mm, df = 0.25 µm
Pneumatics:	Helium, constant flow, 1 mL/min

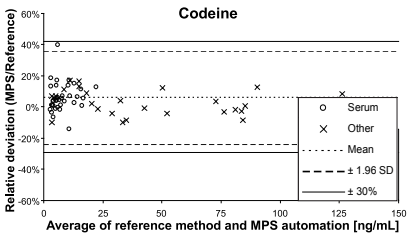
MSD

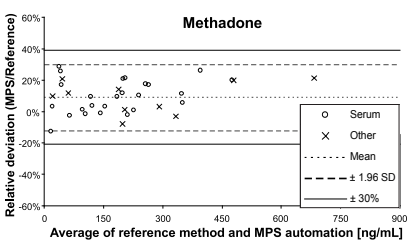
Detection mode:	SIM mode

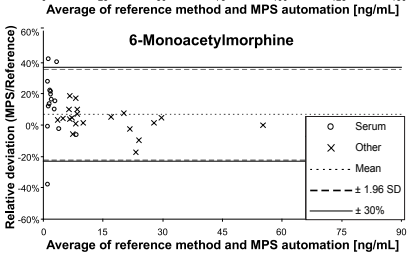












Relative deviations of measured concentrations displayed in Bland-Altman-plots.
ng/mL: Nanogram per milliliter or nanogram per gram for tissue respectively; Other: Other matrices than serum - urine, blood, lyophilized kidney tissue, heart blood, lyophilized and native brain tissue; LOD: Limit of detection; LOQ: Limit of quantification; ULOC: Upper limit of calibration; SD: Standard deviation of relative deviations.

Limit of detection (LOD), limit of quantification (LOQ) and upper limit of calibration (ULOC) for each compound using the validated, partly-automated reference method.

Analyte	LOD [ng/mL]	LOQ [ng/mL]	ULOC [ng/mL]
Cocaine	1.1	3.5	300
Benzoylecgonine	9	47	1500
Methadone	4.2	16.7	1500
Morphine	1.2	4.9	300
Codeine	0.4	2.6	150
6-Monoacetylmorphine	0.3	0.8	150
Dihydrocodeine	0.8	4.2	300
7-Aminflunitranzepam	0.6	2.5	150

Quantifier and qualifier ions for analytes and internal standards.

Compound	Quantifier [m/z]	Qualifier [m/z]
Cocaine	182	303, 198
Cocaine-d_3	185	306, 201
Benzoylecgonine	361	256, 346
Benzoylecgonine-d_3	364	259, 349
Methadone	223	294, 236
Methadone-d_3	226[a], 303[b]	303[a], 318[b], 242
Morphine	429	220, 401
Morphine-d_3	432	223, 404
Codeine	371	234, 343
Codeine-d_3	374	237, 346
6-Monoacetylmorphine	399	340, 400
6-Monoacetylmorphine-d_3	402	343, 403
Dihydrocodeine	373	315, 358
Dihydrocodeine-d_8	379	318, 364
7-Aminflunitranzepam	326[b], 355[a]	326[a], 356[a], 327[b], 354[b]
7-Aminflunitranzepam-d_7	362	333, 363

[a] Qualifier ion used in partly-automated analysis method.
[b] Qualifier ion used in fully automated analysis method.

mL phosphate buffer was aspirated resulting in the final dilution (same as in the reference method). This solution was added to the SPE cartridge and the process was repeated once to transfer the entire sample. The elution volume was reduced from 2 mL to 1.9 mL. The first 0.6 mL was discarded and the last 1.3 mL was collected based on an established elution profile. The derivatization time was shortened from 30 min to 5 min with shaking at 90 °C by employing a mixture of isooctane/pyridine/MSTFA 14/5/1 (v/v/v) instead of the isooctane/MSTFA 19/1 (v/v) mixture originally used.

Close to 170 serum samples and more than 50 samples of other matrices like urine, different tissues and heart blood were analyzed by both methods. Results are equivalent as can be seen in the double logarithmic line- and Bland-Altman-plots. This is true for serum samples and also for alternative matrix samples. Although results between the limit of quantification and the limit of detection may not be reported routinely, they are included in the line plots. Even in this concentration range the method equivalence is obvious. Since only a couple of samples were positive for dihydrocodeine and 7-aminoflunitrazepam these results are not plotted. Samples and quality control samples were also in good concordance for these compounds.

No carryover for any of the compounds could be detected when extracting blank serum after real samples. By overlapping sample preparation steps with the GC/MS run a throughput of around 29 samples per day could be achieved, which is comparable with the partly automated reference method.

The analyses were performed in different laboratories by different personnel at different times proving the ruggedness of the instrumentation and methods and the suitability for routine forensic analysis tasks.

Automated sample preparation

Condition SPE cartridge with 2 mL methanol and 2 mL phosphate buffer (pH 7.9)

Dilute the supernatant of the protein precipitation in the SPE syringe and add the diluted sample to the SPE cartridge

Wash the cartridge with 2 mL water, 2 mL acetic acid, and 2 mL methanol.

Dry cartridge briefly using a flow of nitrogen

Elute with 1.9 mL of dichloromethane/isopropanol/ammonia. The first 0.6 mL are discarded and the following 1.3 mL are collected in a vial

Evaporate the eluate to dryness at 70 °C, 8 kPa and 300 rpm in the *m*VAP station

Reconstitute in 200 µL isooctane/pyridine/MSTFA 14/5/1 (v/v/v)

Shake for 5 min at 90 °C for derivatization

Inject 2 µL into the GC/MS

Calibration solutions were treated analogous to the eluates.

Manual sample pretreatment

All liquids (urine, blood, serum) were treated identically:

1. Protein precipitation by drop-wise addition of a mixture of 0.6 mL sample, 0.1 mL water and 20 µL internal standard solution to a mixture of 1 mL acetonitrile and 0.1 mL isopropanol.
2. Mixing and centrifugation.
3. Transfer of an aliquot (0.75 mL) of the supernatant to individual vials for both analysis methods.

Tissues (brain and kidney, native and lyophilized) were homogenized. An aliquot of approximately 0.6 g was weighed and handled like the liquids above though the acetonitrile/isopropanol solution was added to the sample/standard mixture. The protein precipitation steps could be automated using a centrifuge option with the MPS, but this was not within the scope of this study.

Conclusions

The following achievements were made:

- Comprehensive automation of a validated, partly automated analysis method for opioids, cocaine and metabolites from blood serum and other matrices.
- Analysis results of the methods were found to be equivalent based on GTFCh recommendations.
- The automated method proved to be rugged and suitable for routine analysis in forensic laboratories.
- The automated method saves manual work and reduces the risk of human error. It generates a throughput of 29 samples per day, which is similar to the reference method and is well sychronized with the GC/MS analysis time.
- The analysis system is highly flexible and can reproduce manual sample preparation workflows. Therefore it can be used to automate other validated GC or LC analysis methods or for stand-alone automation of sample preparation.