Highly Sensitive CE-ESI-MS/MS for Accurate Quantitation of Drugs of Abuse in Bioanalysis Using the Agilent 6490 Triple Quadrupole LC/MS System

Application Note

Forensic Toxicology

Authors

Isabelle Kohler, Julie Schappler, and Serge Rudaz
School of Pharmaceutical Sciences
University of Geneva, University of Lausanne
Geneva, Switzerland

Martin Greiner
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

The combination of capillary electrophoresis and mass spectrometry (CE-MS) is particularly well suited to forensic toxicology due to its high separation efficiency, selectivity and sensitivity, short analytical time, and low solvent and sample consumption. A CE-ESI-MS/MS method was developed for the quantitation of drugs of abuse in urine samples with the highly sensitive Agilent 6490 Triple Quadrupole LC/MS system, including Jet Stream and ion funnel technologies, equipped with a triple-tube sprayer especially designed for CE hyphenation. Urines were simply diluted 10-fold prior to CE injection, and a pH-mediated stacking procedure was implemented to increase the loading capacity (20.5 % of the capillary length). This approach was found to increase the sensitivity of the method with limits of detection (LODs) as low as the ng/mL level. The quantitative analytical procedure was validated for two model compounds, cocaine (COC) and methadone (MTD), according to SFSTP protocols and guidance of the Food and Drug Administration (FDA). Performance was evaluated for selectivity, response function, the limit of quantitation (LOQ), trueness, precision, and accuracy. COC was fully validated over a concentration range of 10–1,000 ng/mL, with accuracy included within the ± 30 % tolerance limits, as for MTD in the concentrations range of 21–1,000 ng/mL. The developed CE-ESI-MS/MS was eventually applied to real cases analysis.

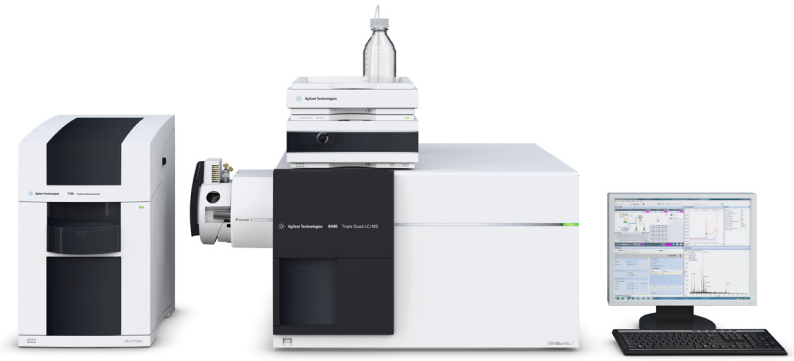
Agilent Technologies

Introduction

The quantitation of xenobiotics in body fluids is important in the field of forensic toxicology. CE represents an alternative technique to GC and LC for a large range of toxicological applications with numerous advantages, such as high separation effi ciency, short analysis time, and low solvent and sample consumption. UV/Vis is the most widely used detection technique with CE confi guration but suffers from a lack of sensitivity due to the narrow optical path length afforded by the internal diameter of the capillary. This lack of sensitivity, combined with the relatively low selectivity of UV/Vis detection, is considered a challenging issue for the determination of potentially low concentrated xenobiotics in body fluids . For quantitative purposes, CE can be hyphenated to various selective MS analyzers such as triple quadrupole in multiple reaction monitoring (MRM) mode. In this study, CE was hyphenated to a highly sensitive 6490 Triple Quadrupole LC/MS system equipped with Jet Stream and ion funnel technologies. Electrospray ionization (ESI) is the most widespread ionization source for coupling CE with MS and was used with the sheath-fl ow configura tion. The sheath-fl ow interface is characterized by an additional make-up liquid fl owing through a so-called triple-tube ESI sprayer that mixes with the CE effl uent at the capillary tip, providing electrical contact at the outlet end, plus the appropriate fl ow rate (µL range) and solvent conditions for ionization of the analytes. A sprayer that presents an adapted design compared to the standard triple-tube sprayer, has been designed and was used for the hyphenation of CE with triple quadrupole.

Triple-Tube ESI Sprayer

The sheath-flow approach has been widely used due its stability, versatility, robustness, and ease-of-use. The Agilent set-up positioned in an orthogonal configuration towards MS entrance derives from the LC/MS configuration. For CE-MS the LC-ESI sprayer has been replaced by a triple-tube sprayer containing two concentric tubes: one tube comprises the CE capillary outlet and transports the sheath liquid, surrounded by the other tube transporting the nebulizing gas. A second generation triple-tube sprayer G1607B has been introduced with modifications of sprayer's tip and needle design that should help position the needle exactly in the center of the sprayer body. Figure 1 illustrates the earlier version CE-MS sprayer G1607A (Figure 1A) and the triple-tube sprayer G1607B (Figure 1B). The performance of both sprayers was evaluated on a set of toxicological compounds at various concentrations, and signal intensities were found to increase up to 1.5 fold with the new triple-tube sprayer, explained by an improvement of spray quality and, therefore, ionization and signal intensity[1].



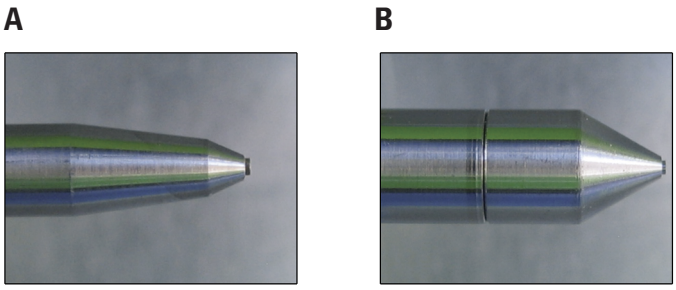


Figure 1. Illustration of CE-ESI-MS sprayer. A) Former triple-tube sprayer (p/n G1607A). B) New Triple-tube sprayer (p/n G1607B).

Agilent Jet Stream Technology

Modifications to the ESI source as well as the sprayer position were carried out to increase ionization and transmission efficiency, with adaptation in the position and number of heated gas inlets, the sprayer orientation relative to the sampling orifice, the diameter of MS orifice, and the number of transfer capillaries[2]. Some of these improvements were envisaged for CE-MS coupling. In this context, Agilent Jet Stream (AJS) thermal gradient focusing technology has been developed to improve (i) desolvation of nebulized spray droplets and (ii) better focus ions while keeping droplets away from the MS orifice. Both effects strengthen signal-to-noise (S/N) ratios and overall sensitivity. AJS consists of a modified ESI source with the addition of a collinear, concentric superheated nitrogen sheath gas surrounding the sprayer, confining the spray plume and increasing the desolvation efficiency. Due to an enhanced ion density in the confinement zone, more ions are available for sampling and, therefore, the overall ionization is more efficient. AJS was previously developed for LC/MS configuration but can be also used for CE-MS hyphenation, only with the G1607B triple-tube sprayer.

In a previous study, a direct comparison of the AJS source versus a conventional ESI source showed comparable performance on a set of low molecular weight compounds in positive ion mode[1].

Agilent iFunnel Technology

iFunnel technology encompasses (i) the AJS technology, (ii) a hexabore capillary, and (iii) a dual-stage ion funnel. On conventional Agilent mass spectrometers, ions produced in the source are initially transferred by a single inlet capillary of *ca.* 600 µm id restricting ion sampling. In a 6490 Triple Quadrupole LC/MS system, a short hexabore capillary assembly is used to increase the interface area of the MS inlet within the thermal ion confinement zone, leading to an increased ion sampling efficiency while maintaining good desolvation performance (Figure 2). Increasing the number of capillaries with the hexabore assembly simultaneously results in an increase of gas load in the mass spectrometer. A dual-stage ion funnel system composed of a series of closely-spaced ring electrodes, as illustrated in Figure 2, is added after the hexabore sampling capillary to remove the gas while increasing ion transmission, reducing neutral contaminants, and decreasing system noise[3,4]. Therefore, this iFunnel technology, which is available on some Agilent mass spectrometers, allows for an increased sensitivity compared to conventional ones.

This Application Note presents the development of a CE-ESI-MS/MS method for the quantitation of drugs of abuse in urine samples with CE hyphenated to the highly sensitive 6490 Triple Quadrupole LC/MS system. Urine samples were diluted 10-fold prior to CE injection and a pH-mediated stacking procedure was implemented to increase the loading capacity (20.5% of the capillary length) with an increase in sensitivity and LODs as low as the ng/mL level. Quantitative procedure was validated for two model compounds, cocaine (COC) and methadone (MTD), according to SFSTP protocols[5] and guidance of the FDA[6]. Performance was evaluated for selectivity, response function, the limit of quantitation (LOQ), trueness, precision, and accuracy. It was eventually applied to real cases.

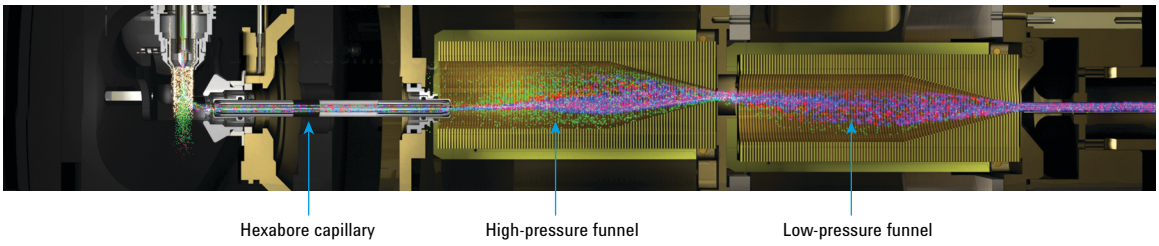


Figure 2. Hexabore capillary and dual-stage ion funnel[4].

Experimental

Chemicals

Hydrochloric acid, sodium hydroxide, analytical grade isopropanol (*i*-prOH), and 28 % ammonia solution (*m/v*) were obtained from Fluka (Buchs, Switzerland). Methanol (MeOH) and formic acid were purchased from Biosolve (Valkenswaard, Netherlands) and were all ULC/MS grade. Ultrapure water was supplied by a Milli-Q Advantage A10 purification system from Millipore (Bedford, MA, USA). Cocaine (COC), deuterated cocaine (d_3-COC), d,l-methadone (MTD) and deuterated d,l-methadone (d_3-MTD) in methanolic solutions (1 mg/mL) were obtained from Lipomed AG (Arlesheim, Switzerland).

Sample Preparation

Blank urine was obtained from a pool of six healthy Caucasian nondrug consumers and stored after collection in polypropylene tubes at –20 °C. Before analysis, the pooled urine was defrosted at ambient temperature, centrifuged at 10,000 rpm for 5 minutes and filtered through a 0.45-µm nylon filter (BGB Analytik AG, Böckten, Switzerland). Stock standard solutions of the solid analytes were prepared by dissolving each compound in MeOH to obtain a concentration of 1 mg/mL and stored at 4 °C until use. Blank pooled urine was spiked daily at desired concentrations. For this purpose, volumes of stock standard solutions were evaporated to dryness under a gentle steam of nitrogen and reconstituted in blank urine. Before injection, urine samples were diluted with BGE and water (1:1:8, *v/v/v*).

Calibration standards (CS) were independently prepared in blank pooled urine on each of the three validation series (j = 3) at three known concentrations (10, 500, and 1,000 ng/mL for both compounds, k = 3) with two replicates for each concentration (n = 2). d_3-COC and d_3-MTD were spiked in each sample at a concentration of 50 ng/mL, and calibration curves were built from the peak areas of COC and MTD *versus* the peak areas of d_3-COC and d_3-MTD, respectively.

Validation standards (VS) were independently prepared in blank pooled urine for each of the three validation series (j = 3) at four known concentrations (10, 25, 500, and 1,000 ng/mL for both compounds, k = 4), with four replicates for each concentration (n = 4). d_3-COC and d_3-MTD were spiked in each sample at a concentration of 50 ng/mL, and reported signals were obtained from the peak areas of COC and MTD *versus* the peak areas of d_3-COC and d_3-MTD, respectively.

Toxicological samples were received from the Laboratory of Clinical Chemistry (Geneva Hospitals, Geneva, Switzerland) and stored at – 20°C until use. Before analysis, samples were treated in the same manner in which the blank pooled urine was treated, and IS were spiked at 50 ng/mL before dilution and injection. Two independent analyses were performed for each sample (N = 2).

Instrumentation

CE experiments were performed with an Agilent 7100 CE system, equipped with an integrated diode array detector, an autosampler and a power supply able to deliver up to 30 kV. Separation was performed using a fused-silica capillary (BGB Analytik AG, Böckten, Switzerland) with a total length of 80 cm and a 50 µm id. Before its first use, the capillary was rinsed sequentially at 2 bar with MeOH (5 minutes), water (3 minutes), 0.1 M HCl (5 minutes), water (3 minutes), 1 M NaOH (5 minutes), and water (3 minutes). The capillary was conditioned daily with MeOH (5 minutes), water (5 minutes), and BGE (10 minutes) at 2 bar. Prior to each sample injection, the capillary was rinsed at 2 bar with BGE (3 minutes). When not in use, the capillary was rinsed with water and dry-stored. Samples were kept at ambient temperature in the autosampler. The CE instrument was coupled to a 6490 Triple Quadrupole LC/MS system through a coaxial sheath flow AJS interface. CE-ESI-MS conditions are presented in Table 1.

Table 1. CE-ESI-MS conditions.

Capillary electrophoresis	
Injection preplug	7 % NH_4OH (*m/v*) at 50 mbar for 10 s (0.7 % of capillary length)
Sample injection	at 100 mbar for 150 seconds (20.5 % of capillary length)
Injection postplug	BGE at 50 mbar for 3 seconds (0.2 % of capillary length)
BGE	1 M formic acid, pH 1.8
Separation voltage	30 kV with initial ramping of 833 V/s (36 seconds)
Temperature	25 °C
Mass spectrometry	
Sheath liquid	*i*-PrOH-water-formic acid (50:50:0.5, *v/v/v*) at 5 µL/min
Drying gas temperature	200 °C
Drying gas flow rate	16 L/min
Nebulizing gas pressure	8 psi
Sheath gas temperature	200 °C
Sheath gas flow rate	3.5 L/min
Nozzle voltage	2,000 V
Capillary voltage	2,000 V
EMV	300 V
Fragmentor voltage	380 V
Dwell time	80 ms
Mass resolution	0.7 u

Table 2 shows MRM transitions for COC, d3-COC, MTD, and d3-MTD with their respective collision energy and cell accelerator voltage.

Results and Discussion

Method Development

In forensic toxicology, a fast, sensitive, and accurate quantitation method is required. In this case, tedious and time-consuming sample preparations, for example, liquid-liquid extraction (LLE), or solid-phase extraction (SPE) procedures are preferably skipped while a simple dilution is promoted if possible. A stacking procedure was implemented to increase the volume injected and to offset the loss of sensitivity caused by urine dilution. In contrast to other stacking procedures, which can be strongly dependent on the saline composition of the sample, a pH-mediated stacking procedure was applied. Samples were diluted 10-fold with BGE and water (1:1:8, *v/v/v)* prior to injection. This dilution allowed for (i) the normalization of urine pH, (ii) a full ionization of analytes before injection, and (iii) a consequent decrease of the sample conductivity. A small preplug of 7 % NH_4OH (*m/v*), corresponding to 0.7 % of the capillary length, was injected prior to acidified diluted urine sample, providing stacking without disrupting the electrophoretic process. When applying the separation voltage, analytes under cationic form migrated until they reached the strong alkaline zone, became neutral, and stacked in a narrow zone at the boundary of the sample and the alkaline plug. The latter was then acidified by the BGE and the analytes returned to a cationic state and began their electrophoretic migration[7]. With the developed pH-mediated stacking procedure, 20.5 % of the capillary length was filled during injection without any peak broadening. Figure 3 illustrates the improvement for COC in loading capacity obtained when injecting 20.5 % of the capillary length with pH-mediated stacking (Figure 3B) compared to the conventional hydrodynamic injection of 1.0 % of the capillary length without sample stacking (Figure 3A). With these conditions, LODs (expressed as the concentration where the S/N ratio was superior to 3) were estimated at 2 ng/mL for MTD and COC.

Table 2. MRM transitions for COC, d_3-COC, MTD, and d_3-MTD.

Compound	Precursor ion	Product ion	Collision energy	Cell accelerator voltage
COC	304.1 *m/z*	182.0 *m/z*	20 eV	3 V
d_3-COC	307.1 *m/z*	185.0 *m/z*	20 eV	3 V
MTD	310.2 *m/z*	265.1 *m/z*	10 eV	3 V
d_3-MTD	313.2 *m/z*	268.1 *m/z*	10 eV	3 V

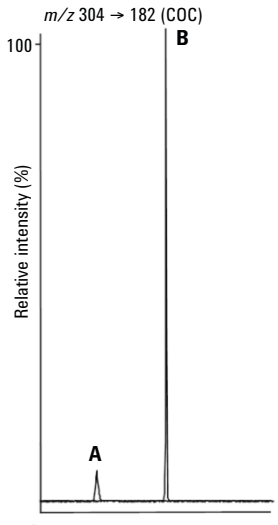


Figure 3. Example of pH-mediated stacking for COC at 100 ng/mL. A) 1% HD injection, without stacking. B) 20.5% HD injection with pH-mediated stacking.

Selectivity

With urine dilution, endogenous compounds that may alter the analyte ionization process (signal suppression or enhancement) are still present. The matrix effect (ME) was evaluated for COC and MTD to determine the influence of potential comigrating interferents on analyte ionization prior to triple quadrupole determination. For this purpose, a procedure based on the methodology proposed by Matuszewski *et al*. for the quantitation of ME in biological fluids was implemented[8]. COC did not show any significant suppression, with an ME of 92 ± 9% (± 2SD), while a relevant signal suppression was observed for MTD, with an ME of 73 ± 5%. Therefore, the use of deuterated IS correction for an external calibration within the reconstituted matrix was selected for quantitation.

The method selectivity was also evaluated by comparing electropherograms obtained by injecting (i) blank urine (CAL 00), (ii) urine spiked with d_3-COC and d_3-MTD at 50 ng/mL (CAL 0), and (iii) a VS at 25 ng/mL for COC and MTD, with the IS set at 50 ng/mL. As illustrated in Figure 4, for both analytes, no interference was detected at the migration times corresponding to COC and MTD or their respective IS.

Validation

SFSTP validation guidelines[5] were followed to evaluate the quantitative performance of the developed CE-ESI-MS/MS method for COC and MTD analysis on three independent series ($j = 3$). The validation protocol involved three concentrations ($k = 3$) with two repetitions ($n = 2$) for CS and four concentrations ($k = 4$) with four repetitions ($n = 4$) for VS. The concentrations' ranges for COC and MTD were determined according to the standard concentrations detected in samples from drug consumers. A concentration range of 10–1,000 ng/mL was selected for both compounds. CS and VS were prepared in blank pooled urine. Several regression models for calibration curve adjustment were evaluated. Trueness (relative bias) and precision were assessed for each concentration level. Precision was estimated with the variances of repeatability (s^2r) and intermediate precision (s^2R), and was expressed by RSD (%). Confidence intervals were calculated with fixed degrees of freedom ($df = k \cdot j - n$) at a risk $\alpha = 5$ %. Accuracy profiles for COC and MTD were built with trueness and upper and lower confidence limits, with the latter two representing the total error of the method.

Response function

Different regression models were assessed for the calibration curve, including:

- Ordinary least square (OLS) regression
- OLS after square root transformation of concentrations (x) and responses (y)
- OLS after logarithm transformation of concentrations (x) and responses (y)
- OLS forced through the origin, external standard with high level of CS, and weighting least square with two weighting factors ($1/x$ and $1/x^2$).

For all of the calibration models, accuracy profiles were plotted for COC and MTD.

The optimal regression model was selected according to the best total error profile obtained when covering the whole concentration range. OLS after square root transformation was selected as the best calibration model for COC with confidence intervals contained inside the acceptance limits, which were set at ± 30% according to the guidelines for quantitation in bioanalysis[9]. For MTD, the best model for calibration was the OLS after square root transformation, but the lowest VS (10 ng/mL) was outside of the acceptance limits. The LOQ was, therefore, established on the basis of the accuracy profile, as discussed below.

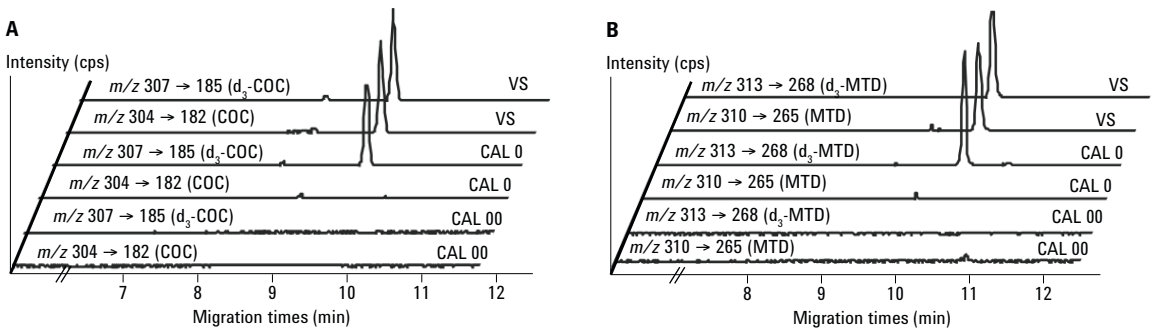


Figure 4. Evaluation of the method selectivity. Electropherograms obtained A) for COC, B) for MTD.

Trueness and precision

The trueness of an analytical procedure expresses the closeness of agreement between the mean values obtained from a series of measurements and the true values[5]. The results for trueness were assessed from the VS by relative bias (%) and are presented in Table 3. For COC, the relative biases were all satisfactory, as they did not exceed the threshold of ± 15 %. For MTD, the relative biases for medium and high concentrations, that is, 25, 500, and 1,000 ng/mL, were lower than ± 2 %. However, with a relative bias of 38.5 %, the lowest concentration (10 ng/mL) was unacceptable.

The precision of the method was estimated using the repeatability and intermediate precision at each VS and was expressed by RSDs. Table 3 shows that the RSD values for COC were in the range of 3.0–5.7 % for both repeatability and intermediate precision, showing strong precision in the developed method. For MTD, unsatisfactory RSD values of 21.0 and 27.8 % were obtained at 10 ng/mL for repeatability and intermediate precision, respectively, while the RSDs were lower than 7.1 % for higher concentrations.

Table 3. Validation criteria and results for COC and MTD in urine (j = 3, k = 4, n = 4).

Validation criterion	COC	MTD
Trueness		
Relative bias (%)		
10 ng/mL	12.0	38.5
25 ng/mL	1.0	1.7
500 ng/mL	-3.1	-1.8
1,000 ng/mL	0.1	0.7
Precision		
Repeatability/intermediate precision [RSD, in %]		
10 ng/mL	5.7/5.7	21.0/27.8
25 ng/mL	5.0/5.0	7.1/7.1
500 ng/mL	4.1/4.1	2.8/3.6
1,000 ng/mL	3.0/3.3	3.0/3.0
Accuracy		
Lower/upper confidence limits of the total errors [%]		
10 ng/mL	-1.1/25.1	-25.5/102.5
25 ng/mL	-10.5/12.4	-14.7/18.0
500 ng/mL	-12.5/6.3	-10.1/6.5
1,000 ng/mL	-7.4/7.6	-6.2/7.6
lower limit of quantitation LLOQ (ng/mL)	10	21

Accuracy

Accuracy is the expression of the total error of the analytical method and was chosen to evaluate the capacity of the developed analytical method to quantify samples with an accepted risk of α = 5 %[6,10]. The relative accuracy profiles for COC and MTD are shown in Figure 5. The lower and upper confidence limits of the mean bias (%) for COC, as shown in Table 3, were included within the acceptance limits of ± 30 % for each level of concentration. The developed method is, therefore, accurate for the quantitation of COC over the investigated concentration range (10–1,000 ng/mL). The lowest concentration level (10 ng/mL) was confirmed to be the LLOQ, which is defined by the smallest quantity of analyte that can be quantified with a defined accuracy within the acceptance limits.

For MTD, the LLOQ was interpolated from the absolute accuracy profile of MTD and defined at 21 ng/mL because the lowest concentration level (10 ng/mL) was not included within the acceptance limits of ± 30 %. With this LLOQ, the quantitation of MTD was found to be accurate in the range of 21–1,000 ng/mL.

Application to Real Cases

COC and MTD contained in two toxicological samples coming from the Laboratory of Clinical Chemistry (Geneva Hospitals, Switzerland) were quantified with the developed method. A calibration curve was constructed the same day (k = 3, n = 2) and OLS was applied after square root transformation of concentrations and responses. The confidence interval associated to the mean results is expressed with Equation 1.

Equation 1.

$$\bar{x} \pm t_{df,\alpha} \sqrt{\frac{s^2r}{N} + s^2g}$$

$\bar{x}$ is the mean result and N is the number of analyses. $t_{df,\alpha}$ (Student constant dependent on α and df), s^2r and s^2g were determined during a validation with regular ANOVA-based variance decomposition. Because most of the variability came from repeatability (s^2r) and not from the interseries variance (s^2g), two replications (N = 2) were performed to reduce the intraday variability and to obtain a narrow confidence interval for the final result. In the first sample, COC concentration was found to be 41.0 ± 6.4 ng/mL.

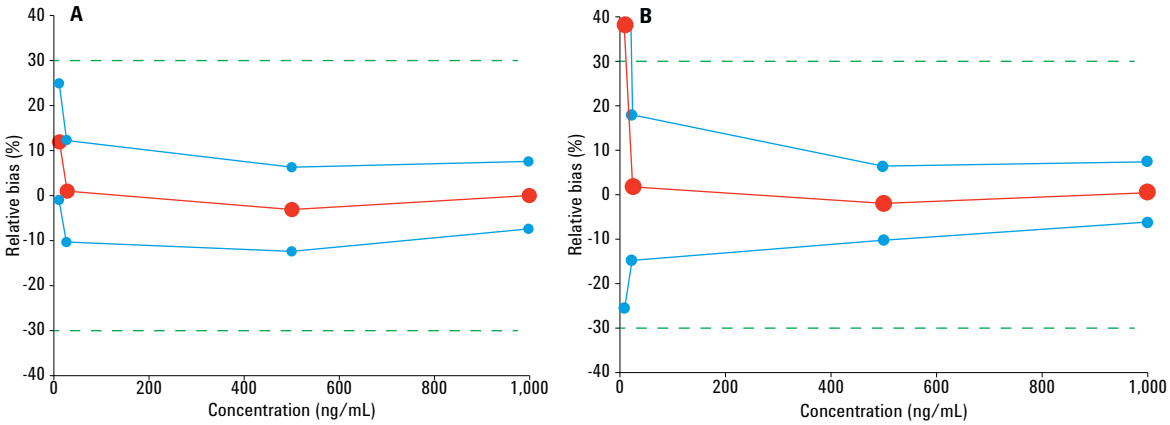


Figure 5. Accuracy profiles obtained for A) COC, and B) MTD in urine. Green dashed lines express ± 30 % acceptance limits

Figure 6A presents the corresponding electropherograms. This relatively low concentration can be related to a low dose (for example, less than ca. 10 mg of crack, intranasal, or intravenous dose) and/or a late urine collection (> 24 hours) after COC consumption. In the second sample, 462.9 ± 33.5 ng/mL of MTD were detected. Figure 6B shows the electropherograms obtained for the second sample. Due to the relatively long detection time window of MTD in urine and the high CYP450 inter-individual, this concentration can be related to both initial and maintenance MTD treatment.

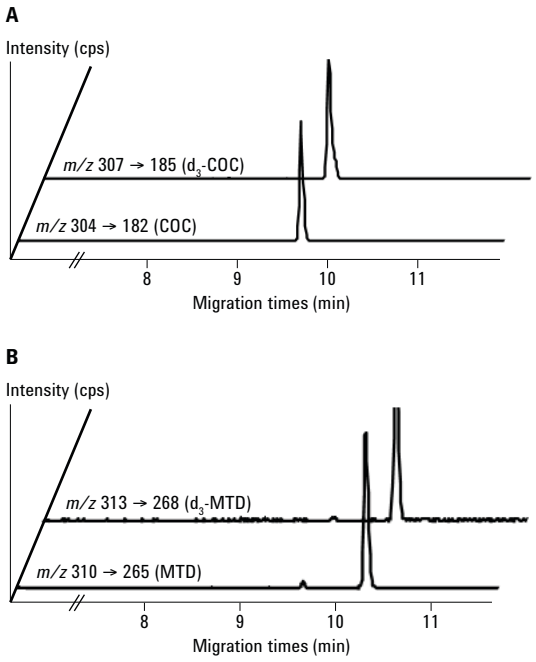


Figure 6. Electropherograms obtained for toxicological samples. A) Sample no. 1 containing COC, B) Sample no. 2 containing MTD.

Conclusion

A fast, selective and sensitive CE-ESI-MS/MS method was developed for the quantitation of drugs of abuse in urine with the highly sensitive Agilent 6490 Triple Quadrupole LC/MS system equipped with a triple-tube sprayer, as well as Jet Stream and ion funnel technologies. Urine samples were diluted 10-fold prior to CE injection, avoiding a tedious and time-consuming sample preparation, and a pH-mediated stacking procedure was implemented to increase the loading capacity (20.5% of the capillary length). The combination of this online preconcentration with the highly sensitive 6490 Triple Quadrupole LC/MS system led to LODs as low as the ng/mL level in urine.

A validation procedure based on accuracy profiles was applied to assess the quantitative performance of the developed CE-ESI-MS/MS. Selectivity, response function, the LLOQ, trueness, precision, and accuracy were estimated for two model and common drug of abuse, COC and MTD. COC was fully validated over the range of concentrations of 10–1,000 ng/mL with accuracy included within the ± 30% tolerance limits, as for MTD in the concentrations range of 21–1,000 ng/mL.

The developed CE-ESI-MS/MS method was successfully applied to real toxicological samples.

References

1. I. Kohler, *et al.*, Agilent Technical Note, publication number 5990-9716EN, **2012**.
2. H. Stahnke, *et al.*, *J. Mass Spectrom.*, 47, 875-884, **2012**.
3. R.T. Kelly, *et al.*, *Mass Spectrom. Rev.,* 29, 294-312, **2010**.
4. P. Momoh, *et al.*, Agilent Technical Note, publication number 5990-5891EN, **2010**.
5. P. Hubert, *et al.*, *J. Pharm. Biomed. Anal.*, 45, 70-81, **2007**.
6. http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf (07.01.2013).
7. O.A. Mayboroda, *et al.*, *J. Chromatogr. A*, 1159, 149-153, **2007**.
8. B.K. Matuszewski, *et al.*, *Anal. Chem.*, 75, 3019-3030, **2003**.
9. C.T. Viswanathan, *et al.*, *Pharm. Res.*, 24, 1962-1973, **2007**.
10. E. Rozet, *et al.*, *Trends Anal. Chem.*, 30, 797-806, **2011**.

www.agilent.com/chem/ce

For Forensic Use Only.

This information is subject to change without notice.

Published in the USA, July 1, 2013
5991-2395EN

Agilent Technologies