Evaluation of Mixed-Mode Ion Exchange SPE for the Extraction of Opioids from Human Urine by UHPLC-MS/MS

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Background

The goal of any solid phase extraction (SPE) or sample preparation method development is to obtain the best analyte recovery while minimising the concentration of contaminating compounds reaching the final analysis sample. Traditional loose-filled SPE products are susceptible to a wide range of technical issues arising from poor packing of the resins into cartridges. Inconsistent packing can be problematic during method development resulting in voiding and channelling thereby increasing the risk of obtaining poor analyte recoveries.

The Microlute™ CP SPE range eliminates loose packing in the manufacturing process and therefore, overcomes these technical issues often experienced during sample preparation. Instead it consists of a unique, solid hybrid polymer structure composed of an interconnected network of evenly distributed pores immobilised with the retentive media. This design enhances the flow-through of samples to maximise interactions between analytes and the solid phase to deliver a highly reproducible SPE method.

This application describes the SPE LC-MS/MS methodology using a strong cation exchange (SCX) SPE 30 mg 96 well microplate for the determination of 12 opioids from human urine. It also examines the performance benefits of a hybrid polymer product versus a loose packed plate.

Introduction

The Opioid Crisis

Opioids have been viewed as some of the most effective drugs for pain relief and classed as an essential part for reducing suffering of patients [1]. Due to their effectiveness in pain relief, this class of drugs has caused a lot of controversy due to their administration for recreational purposes. This has led to a normalisation of their use, leading to widespread addiction and abuse of their use all over the world. Europe has been estimated to have had 1.3 million high risk users in 2017 [2] and the United States had an estimated 10.3 million people aged 12 or older misusing opioids in 2018 [3].

With this increase in abuse of opioids, comes a greater importance in laboratory testing for the presence of opioids to help detect patients misusing opioids while still allowing other patients to get the pain relief they need. Laboratory testing has a key part to play to help with solving the opioid crisis in the United States. Quest diagnostics (a large national clinical U.S laboratory) analysed their drug testing data from 2011 - 2017 which contained 3.9 million de-identified drug monitoring tests. This reported findings that in every year from 2011 - 2017, the majority of tests performed were classed as inconsistent with the expected result for their prescribed amount [4]. Therefore it is possible to conclude from these results that testing is needed to confirm if a patient is abusing their prescriptions or using other illicit drugs with their prescription. Chromatographic urine drug testing is there to allow a definitive verification if a patient is using their opioids as prescribed or disregarding their plan and leading towards abuse [5].

Detection of Opioids

Two tests are currently used for detection of opioids in urine: an immunoassay screening test and a chromatographic test. The standard opioid immunoassays are typically designed to detect the natural morphine-like molecules (morphine and codeine) but do not detect synthetic opioids like fentanyl and methadone [6]. The advantages of using chromatographic techniques is that it is possible to identify all the different opioids in one method to target different classes. It also provides a quantifiable result on each opioid present in the sample instead of the qualitative immunoassay result.

Other techniques for determining opioids in urine include a chromatographic 'dilute and shoot' method [7, 8], this involves diluting a sample with an internal standard solution and injecting straight onto LC-MS system. However, compared to a solid phase extraction (an SPE) method, it does not allow concentration of a sample. With low levels of opioids often present in urine samples this could result in false negatives, especially so on opioids that do not give a strong response on a mass spectrometer. SPE also removes any matrix components from the urine sample which are present in a dilute and shoot method. These can affect ionisation of the opioids and build up in the MS source resulting in unreliable

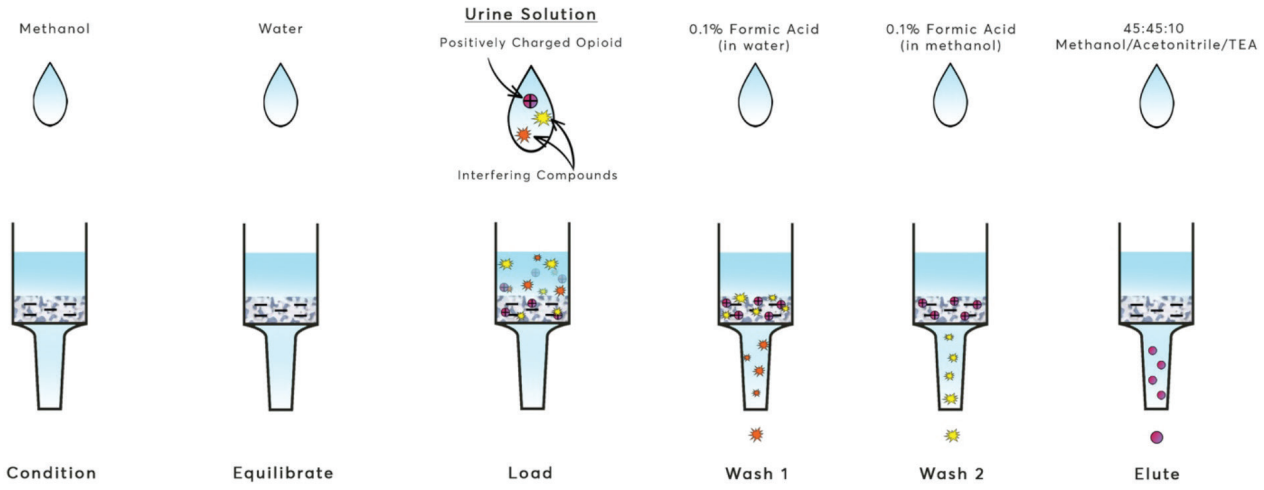

Figure 1. Schematic protocol for sample preparation using Microlute™ CP SCX 96 well plate.

results. This creates longer lead times for testing, expensive re-tests and an increase instrument maintenance time.

This application note demonstrates how an SCX mixed-mode SPE method combined with UHPLC-MS/MS can analyse 12 natural, semi-synthetic and synthetic opioid drugs and metabolites from a sample of human urine in a reliable and reproducible way.

Compounds:

Morphine, oxymorphone, noroxycodone, hydrocodone, norhydrocodone, O-desmethyl-cis-tramadol, norfentanyl, cis-tramadol, meperidine, fentanyl, EDDP, methadone. All compounds were purchased from Cerilliant in their 1 mg/mL format.

Sample Preparation:

A stock of all opioids (3 µg/mL) was prepared in methanol. For the preparation of the urine samples, 12.5 mL of pooled source mixed gendered blank human urine was spiked with 625 µL of opioid stock solution. Blanks were prepared by spiking 12.5 mL of urine with 625 µL of methanol. The samples and blanks were both diluted 1:1 with 1% formic acid in water and vortexed for one minute to homogenise the solutions.

For the mixed-mode SPE, each well of the Microlute™ CP SCX 30 mg 96 well plate (Cat no. PSCX030P-001) was conditioned with 1,000 µL of methanol then equilibrated with 1,000 µL of water. Each pre-treated sample and blank was loaded onto the plate in full. Once loaded, 1,000 µL of 0.1% formic acid in water was used to wash each well, followed by 1,000 µL of 0.1% formic acid in methanol. Each well was eluted with 2 x 400 µL of 45:45:10 methanol/acetonitrile/TEA. The eluent was evaporated under N_2 to dryness using the MiniVap® Gemini (Cat no. 500234) using the 96 needle head with straight needles (Cat no. 229036) at room temperature, taking approximately 45 minutes. Samples were reconstituted in 100 µL of starting mobile phase (0.1% formic acid in water). Post spike standards were prepared by reconstituting the blank solution wells with 100 µL of opioids standard solution (1500 ng/mL) diluted in starting mobile phase (0.1% formic acid in water). The same procedure was also performed on a commercially available loose filled 96 well plate (Figure 1).

Experimental

LC Conditions:

LC system	Vanquish Horizon
Column	Thermo Scientific™ Hypersil GOLD™ aQ, 3 µm, 100 x 4.6 mm
Column temp.	30°C
Injection volume	4 µL
Flow rate	1 mL/min
Mobile phase A	0.1% Formic acid in water
Mobile phase B	0.1% Formic acid in methanol
Gradient	

Time (min)	A%	B%
0.0	100	0
3.0	85	15
8.0	0	100
9.0	0	100
9.1	100	0
15.0	100	0

MS Conditions:

MS system	TSQ Altis
Ionisation Mode	H-ESI
Acquisition Mode	MRM
Polarity	Positive
Spray Voltage	+3500 V
Sheath gas	60 arb
Aux gas	2 arb
Sweep gas	2 arb
Ion transfer tube temp.	380°C
Vaporiser temp.	350°C
Cycle time	0.3 seconds
Q1 resolution	0.4 FWHM
Q3 resolution	0.4 FWHM
CID gas	1.5 mTorr

Results and Discussion

Chromatography

A chromatogram of all the compounds from the 150 ng/mL calibration standard is shown in Figure 2. The peak assignments for the chromatogram can be found in Table 1.

The LC method starts with a 100% water solution to ensure that salts and the most polar components that may still be present in the injected solution are eluted at the beginning of the run before any of the opioids are eluted from the column. This prevents those compounds from interfering with ionisation of the opioid compounds.

The first opioid peak to elute is morphine at a time of 3.60 minutes and the last peak is methadone at 7.71 minutes. All peaks were resolved apart from hydrocodone and its metabolite norhydrocodone (compounds 4,5). Due to the differing precursor ions, this means there is no interferences with the signals.

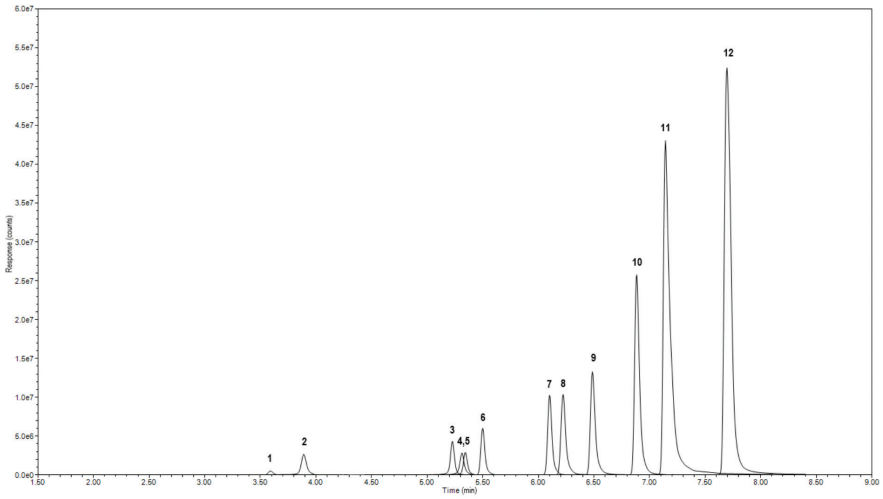

Figure 2. Chromatogram of 150 ng/mL calibration standard of opioids and metabolites. Peak assignments can be found in Table 1.

The 12 analysed opioids and metabolites are listed in Table 1. There is variety of polarities across the range of compounds - the most hydrophilic compound at a LogP value of 0.70 for noroxycodone and the most hydrophobic at a LogP value of 5.20 for EDDP. All the compounds analysed are weak bases with pKas ranging from 8.2 - 10.1 which means the SCX (Strong Cation Exchange) resin is the best suited SPE resin to capture and produce clean samples to inject onto an LC-MS system.

Table 1. Properties and MS parameters for the compounds analysed - [a] Predicted value from Drugbank [9] [b] Predicted value from Pubchem [10], [c] Predicted value from The Metabolomics Innovation Centre [11].

Number	Compound	Opioid Class	R.T (min)	Formula	Molecular Mass	LogP	pKa]	MRM Transitions	Coll. Energy (V)
1	Morphine	Natural	3.60	$C_{17}H_{19}NO_3$	285.14	0.87[a]	8.2[a]	286.13>165.1 286.13>201.1	40.92 25.55
2	Oxymorphone	Semi-synthetic	3.90	$C_{17}H_{19}NO_4$	301.13	0.83[a]	8.2[a]	302.17>227.1 302.17>284.2	27.83 19.39
3	Noroxycodone	Semi-synthetic metabolite	5.23	$C_{17}H_{19}NO_4$	301.13	0.70[b]	9.5[a]	302.1>227.1 302.1>284.1	28.32 15.76
4	Hydrocodone	Semi-synthetic	5.32	$C_{18}H_{21}NO_3$	299.15	2.20[b]	8.6[a]	300.1>171.1 300.1>199.1	38.69 29.42
5	Norhydroco-done	Semi-synthetic metabolite	5.35	$C_{17}H_{19}NO_3$	285.14	1.70[b]	10.1[a]	286.1>171.1 286.1>199.2	36.71 27.50
6	O-desmethyl-cis-tramadol	Synthetic metabolite	5.50	$C_{15}H_{23}NO_2$	249.17	2.30 [b]	9.0[a]	250.1>56.1 250.1>58.1	41.00 16.67
7	Norfentanyl	Synthetic metabolite	6.11	$C_{14}H_{20}N_2O$	232.16	1.60[b]	10.0[c]	233.1>84.1 233.1>177.1	17.22 15.11
8	Cis-tramadol	Synthetic	6.23	$C_{16}H_{25}NO_2$	263.19	1.34[a]	9.4[a]	264.1>56.1 264.1>58.1	46.00 16.09
9	Meperidine	Synthetic	6.49	$C_{15}H_{21}NO_2$	247.16	2.72[a]	8.6[a]	248.1>174.2 248.1>220.2	19.41 20.88
10	Fentanyl	Synthetic	6.89	$C_{22}H_{28}N_2O$	336.22	4.05[a]	9.0[a]	337.1>105.1 337.1>188.2	37.03 21.99
11	EDDP	Synthetic metabolite	7.15	$C_{20}H_{23}N$	277.18	5.20[b]	9.6[c]	278.1>234.1 278.1>249.2	30.31 22.88
12	Methadone	Synthetic	7.71	$C_{21}H_{27}NO$	309.21	3.93[a]	9.2[a]	310.1>105.1 310.1>265.2	26.90 14.74

Recovery and Reproducibility

This application used a reconstitution volume of 100 µL which lead to a 10 times concentration of the original 1 mL of urine loaded onto the plate. This allowed for greater sensitivity of every compound analysed. The process of the SPE helped to create a cleaner solution by binding the analytes to the resin while washing away interference compounds present in the urine matrix - a high aqueous wash to clean off any polar compounds and a methanol wash to elute the hydrophobic acidic and neutral interferences. These two washes help create a very clean solution when samples are eluted from the SPE product as minimal interferences should still be present when the elution step is performed.

Recoveries for each of the 12 compounds spiked into the human urine were measured after sample preparation was performed using the Microlute™ CP SCX 96 well plate. Figure 3. shows the average recovery, from urine samples spiked pre-sample preparation, of all the compounds from a sample size of 12 replicates. All recoveries for the opioids were greater than 80% except for meperidine and EDDP.

Recoveries were compared to a commercially available 30 mg loose filled product using the same method as run on the Microlute™ CP SCX plate for a comparison. The comparison of the two products are found in Figure 4. The recovery for the loose filled product showed a decrease in recovery across each analyte when compared to the hybrid polymer. This reduction is proposed to be due to the structure of the hybrid polymer which provides more efficient interactions between the analyte and chromatographic media. As a result, there is an overall reduction of channelling effects of analyte and solvent throughout the Microlute™ CP SCX plate.

Channelling is the process of liquids taking the path of least resistance through the SPE bed. Therefore, when urine is loaded onto the plate it may not fully interact with all of the resin present due to the uneven flow path through the SPE bed. This effectively reduces the loading capacity of the product and can lead to breakthrough of analyte leading to low recoveries. The second issue is that on elution, solvent has less contact with the bound analytes which can lead to lower recoveries and a need for larger elution volumes in comparison to the hybrid polymer's elution volumes.

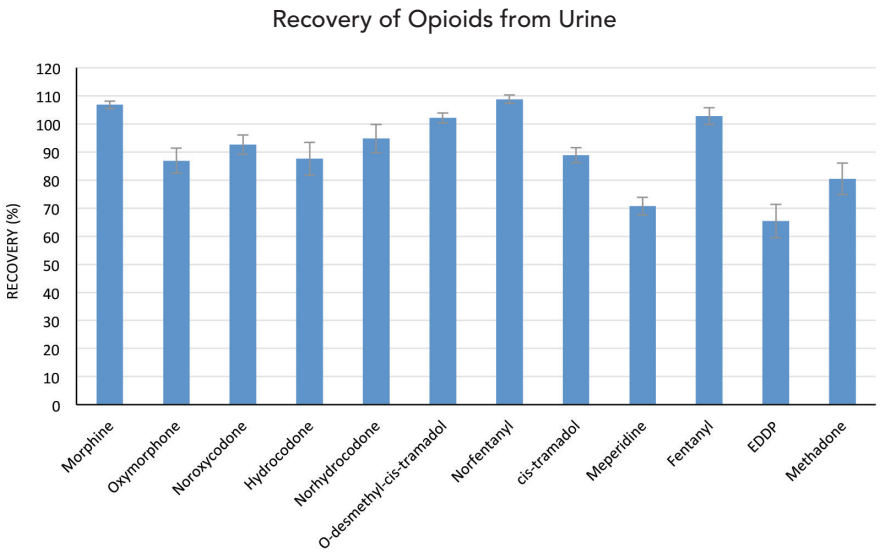

Figure 3. Mean recovery of opioid compounds from human urine using Microlute™ CP SCX plates. Error bars represent the standard deviation of the recoveries (n=12).

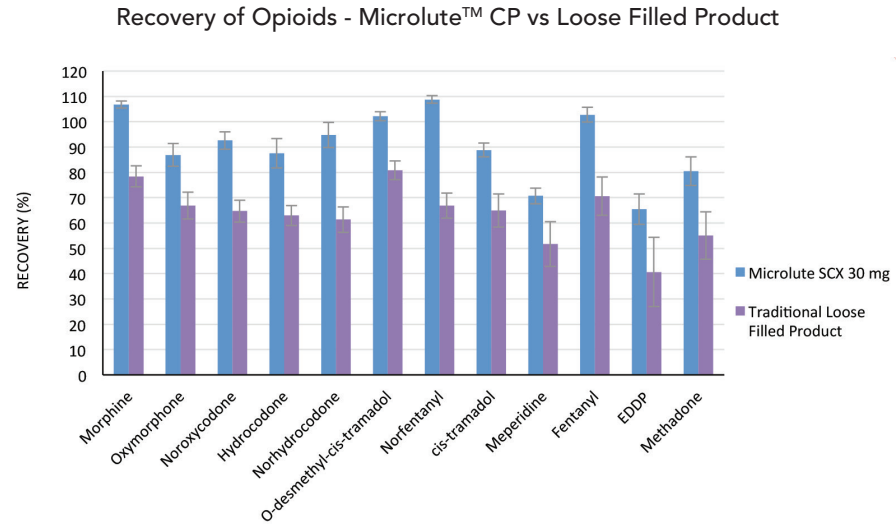

Figure 4. Comparison of recovery of Microlute™ CP SCX 30 mg to a loose filled 30 mg SCX product. Error bars are standard deviation of the recovery results (n=12).

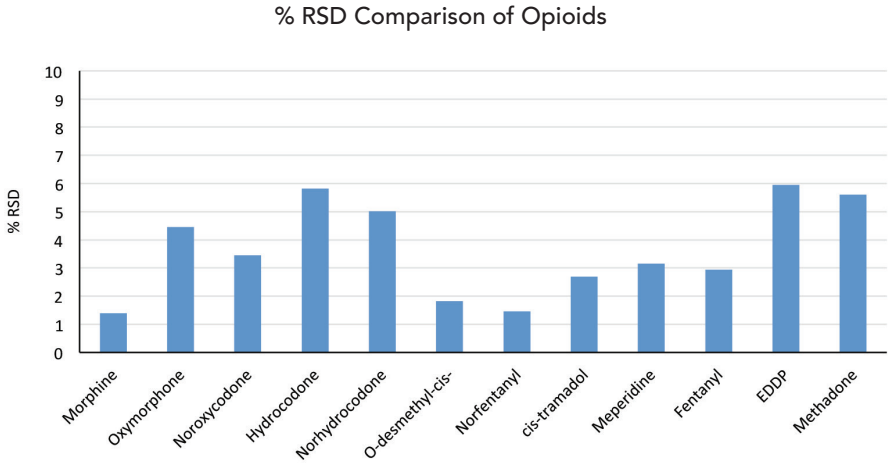

Figure 5. % RSD of all 12 opioids and metabolites calculated from recovery of analytes (n=12).

Well-to-well reproducibility is a measure of how close each recovery result is to each other between different wells of the 96 well plate. It is an important measure to allow confidence in results collected. When a low reproducibility is recorded it can bring doubt into the results collected. For method validation, it is an important metric to look at with suggested guidelines in place for what level is acceptable. Chromatographic

bioanalytical assays typically have a guideline of <15 % RSD or <20% if at the LOD or LOQ level [12, 13, 14] to validate a method. This is a very important measure for a test in which you need confidence in like an opioid test which can have large consequences for the patient who has been tested.

Figure 5 shows the % RSD of the recovery results. The data shows % RSD values ranging from 1.39% for morphine to 5.95% for EDDP. These figures show that the reproducibility of those 12 replicates are falling well within the typical suggested limits for % RSD guidelines often chosen for method validation.

Summary

Microlute™ CP SCX is able to detect a wide range of opioids in urine including natural, semi-synthetic and synthetic compounds making it the obvious choice for drugs of abuse analysis. The process of SPE allows for concentration of samples to allow for more sensitive analysis of lower concentration samples. This is not possible for 'dilute and shoot' methods due to the core principle of that method is to dilute down the sample. The Microlute™ CP SCX "microplate also offers advantageous recoveries across the range of different classes of opioids with high reproducibility <10 % RSD values. This ensures the product gives reliable and reproducible results which is an important metric in drugs testing where confidence in the data output is required.

References:

1. Rosenblum, A., Marsch, L., Joseph, H. and Portenoy, R., 2008. Opioids and the treatment of chronic pain: Controversies, current status, and future directions. Experimental and Clinical Psychopharmacology, 16(5), pp.405-416.
2. European Monitoring Centre for Drugs and Drug Addiction, 2019. European Drug Report: Trends And Developments. [online] Available at: https://www.emcdda.europa.eu/system/files/publications/11364/20191724_TDAT19001ENN_PDF.pdf
3. Substance Abuse and Mental Health Services Administration (SAMHSA), 2018. Key Substance Use And Mental Health Indicators In The United States: Results From The 2018 National Survey On Drug Use And Health. [online] Available at: https://www.samhsa.gov/data/sites/default/files/cbhsq-reports/NSDUHNationalFindingsReport2018/NSDUHNationalFindingsReport2018.pdf
4. Quest Diagnostics, 2018. Health Trends™ Drug Misuse In America 2018: Diagnostic Insights From Clinical Drug Monitoring Into The Opioid Epidemic. [online] Available at: https://www.questdiagnostics.com/dms/Documents/drug-prescription-misuse/Health_Trends_Report_2018.pdf
5. Mahajan, G., 2017. Role of Urine Drug Testing in the Current Opioid Epidemic. Anesthesia & Analgesia, 125(6), pp.2094-2104.
6. Consensus Statement. Appropriate Use of Drug Testing in Clinical Addiction Medicine. Journal of Addiction Medicine, June 2017. [online] Available at: https://www.asam.org/docs/default-source/quality-science/appropriate_use_of_drug_testing_in_clinical-1-(7).pdf
7. Dams, R., Murphy, C., Lambert, W. and Huestis, M., 2003. Urine drug testing for opioids, cocaine, and metabolites by direct injection liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry, 17(14), pp.1665-1670.
8. Edinboro, L.E., R.C. Backer, and A. Poklis, Direct Analysis of Opiates in Urine by Liquid Chromatography-Tandem Mass Spectrometry. Journal of Analytical Toxicology, 2005. 29(7): pp. 704-710.
9. Drugbank.ca. 2020. Drugbank. [online] Available at: https://www.drugbank.ca
10. Pubchem.ncbi.nlm.nih.gov. 2020. Pubchem. [online] Available at: https://pubchem.ncbi.nlm.nih.gov/
11. Hmdb.ca. 2020. Human Metabolome Database. [online] Available at: https://hmdb.ca
12. Food and Drug Administration (FDA), 2018. Bioanalytical Method Validation Guidance For Industry. [online] Available at: https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf
13. European Medicines Agency (EMA), 2019. ICH Guideline M10 On Bioanalytical Method Validation. [online] Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/draft-ich-guideline-m10-bioanalytical-method-validation-step-2b_en.pdf
14. Karch, S., 2007. Workplace Drug Testing. Boca Raton, FL: CRC Press, p.67