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Application News

No. AD-0231

Toxicology / LC/MS/MS & CLAM-2000

Development of Multi-target Screening Method for Toxicological Compounds in Blood Samples on A Fully-automated CLAM-LC-MS/MS Platform

❑ Introduction

Multi-target screening by LC/MS/MS has been widely adopted in detection and quantitation of drugs of abuse (DoA) in forensic investigation and toxicological research [1]. A wide range of targets screened includes illicit drugs, narcotics, psychotropics, antipsychotics, pharmaceuticals and other toxic compounds in urine, serum/plasma and whole blood samples. Sample preparation is often a bottleneck due to the tedious steps. It is also a factor responsible for inaccurate or false negative results. We describe a solution by using an automated sample preparation module CLAM™ -2000 coupled to LC/MS/MS system (LCMS™-8060) for multi-target screening of 61 drugs in whole blood. A ready-to-use method package Rapid Toxicology Screening [2] was used to set up the screening method with human whole blood (frozen) spiked sample without efforts in LC and MRM method development.

❑ Experimental

The 61 targeted drugs (see Table 2) with 26 deuterated drugs as internal standards (IS) were analysed on a high throughput analysis platform, which consists of CLAM-2000 coupling with the LCMS-8060 triple quadrupole system. Automated sample preparation process was carried out on the CLAM-2000 module involving pre-programed steps: wetting of filtering vial with solvent, blood sample dispensing (50 µL), acetonitrile dispensing (250 µL), stirring for 60 seconds at 2000 rpm, filtering for 90 seconds and vial transferring to autosampler. Co-injection (5 µL sample + 20 µL water) mode was adopted on a SIL-30AC autosampler for reducing solvent effect and improving peak shape. The whole procedure was run automatically for a whole batch run including solvent, calibrants, blank, blood samples (spiked), QC samples.

Table 1. Analytical conditions on LCMS-8060

Column	C18 (2.1 mm I.D. x 100 mmL., 2.6µm)
Flow rate	0.3 mL/min
Mobile phase	A: 10 mM of ammonium formate, with 0.1% formic acid B: 10 mM of ammonium formate, with Methanol and 0.1% formic acid
Elution mode	0 min: 5% B → 2 min, 15% B → 10 min, 50% B → 12-20 min, 95% B → 20.1-26, 5% B (end)
Oven temp.	40°C
Injection vol.	5.0 µL
Interface	ESI (heated)
MS mode	Positive, MRM
Interface temp.	300°C
DL temp.	250°C
Heat block temp.	400°C
Nebulizing gas	N_2, 3 L/min
Drying gas	N_2, 10 L/min
Heating gas	Purified Air, 10 L/min

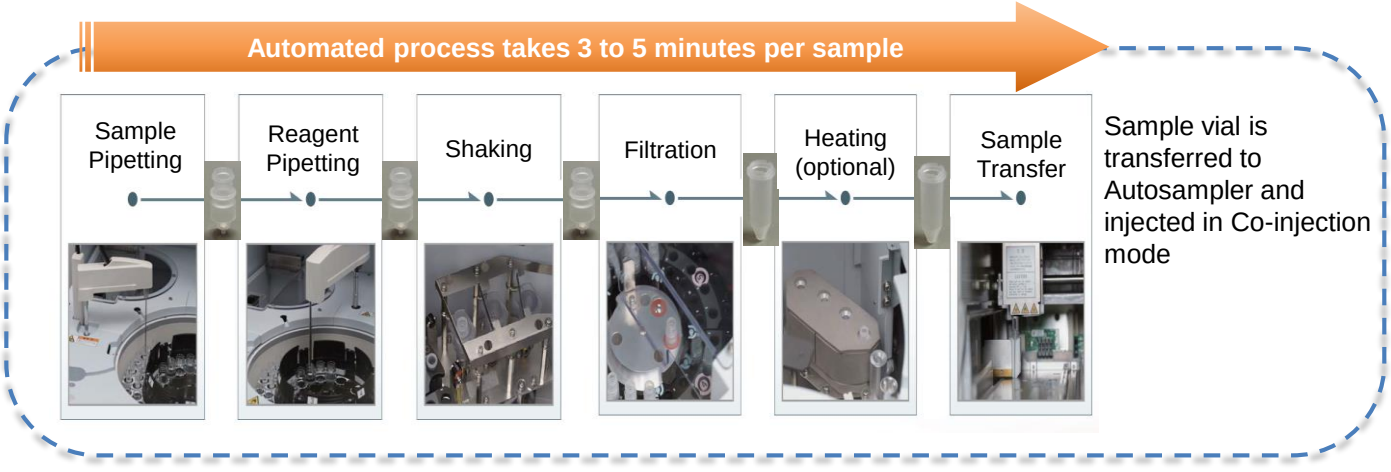


Figure 1. Workflow of CLAM-2000 for automated sample preparation coupled with LCMS-8060

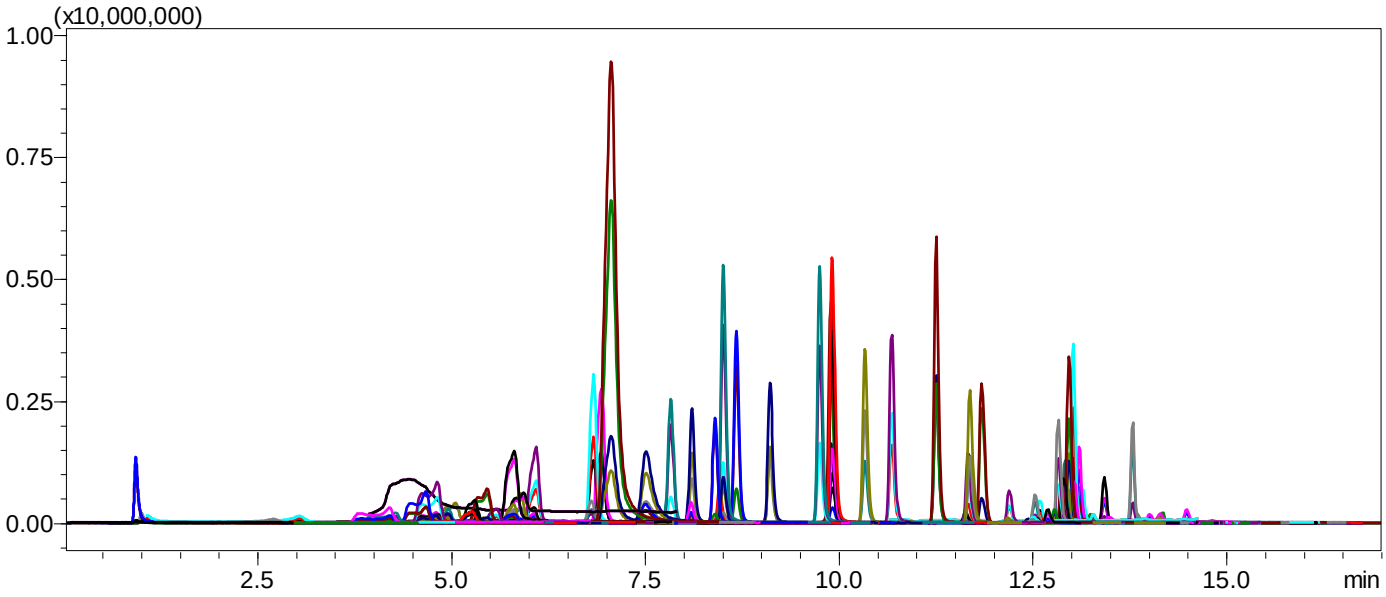


Figure 2: MS chromatogram of mixed standards of 61 targeted drugs (each 20 ppb) with 26 IS (each 4.0 ppb).

A clean human whole blood sample was thawed from deep frozen storage and was used as blank and the matrix for preparation of spiked samples for evaluation of the method performance. Following the method package LC conditions [2], the chromatographic separation of the drugs was achieved with a gradient elution in 26 minutes (See Table 1). Analysis of batch screening data was carried out using LabSolutions Insight version 3.5.

❑ Results and Discussion

MRM method for analysis of 61 DoA

An MRM method with 2-3 optimized MRM transitions for each compound was used directly from the method package of Rapid Toxicology Screening (161 toxicological compounds) [2]. Retention times of the compounds were updated with mixed standards. MRM optimization of some deuterated internal standards were carried out and the collision energies were added to the method. A representative chromatogram of the 61 targets with 26 deuterated IS is shown in Figure 2.

For the 61 targets, only 26 deuterated internal standards (d3~d10) were available. These 26 drugs each with IS are remarked with w.IS in Table 2. The other 35 compounds were screened and quantified with the respective IS which retentions are the closest to the targets. Linear calibration curves were established and good linearity with R2 >/= 0.99 was obtained for the 61 targets with three calibration levels of 4, 20 and 100 ppb and IS at 4 ppb (Table 2). Representative calibration curves are displayed in Figure 3.

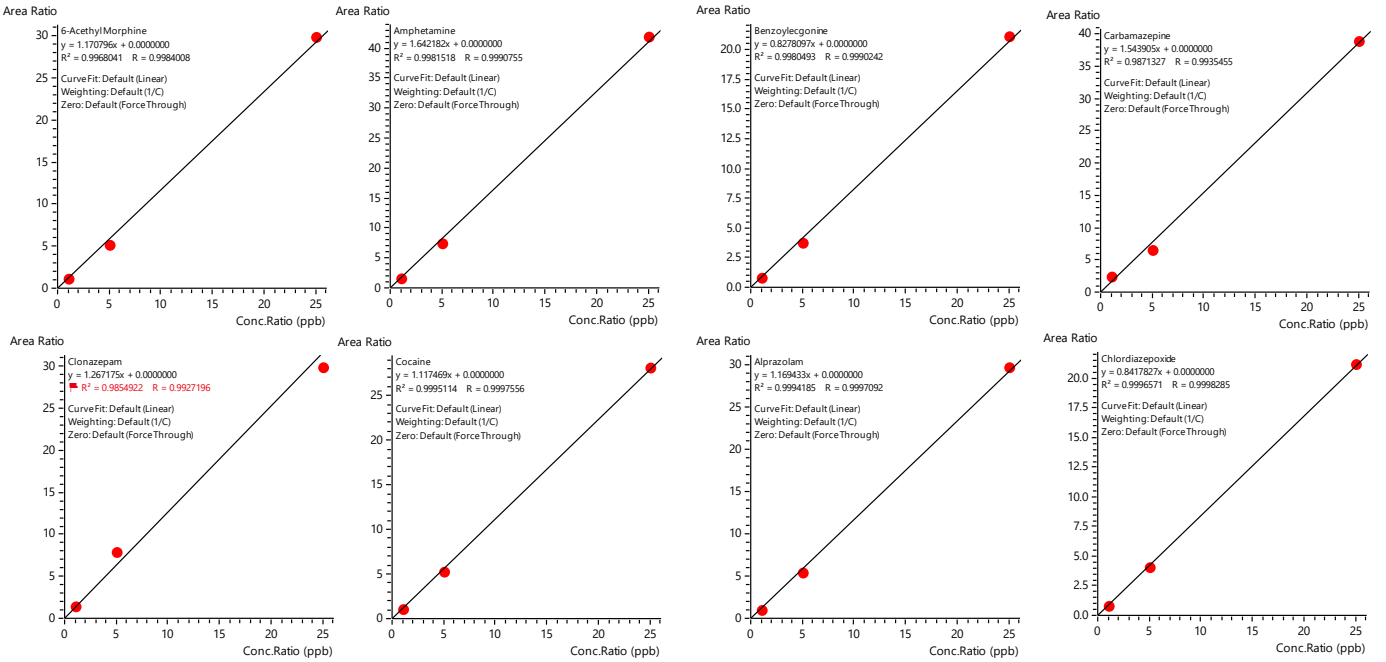


Figure 3: Representative calibration curves of mixed standards (4, 20 and 100 ppb) with IS (4.0 ppb). The drug names are referred to SN 1 ~ SN 8 in Table 2.

Table 2. Analytical conditions of screening of 61 targeted drugs (DoA) on CLAM-2000 / LCMS-8060

SN	Compound Name	Ret. Time (min)	MRM Quantifier (m/z)	ISTD Group	Calibration range (ppb)	R^2
1	6-Acethyl Morphine (w.IS)	5.83	328.0>165.0	1	4-100	0.9984
2	Amphetamine (w.IS)	5.48	136.1>91.1	2	4-100	0.9981
3	Benzoylecgonine (w.IS)	7.53	290.2>168.2	3	4-100	0.9981
4	Carbamazepine (w.IS)	12.54	237.1>194.1	4	4-100	0.9875
5	Clonazepam (w.IS)	12.56	316.1>270.1	5	4-100	0.9855
6	Cocaine (w.IS)	8.51	304.2> 182.2	6	4-100	0.9995
7	Alprazolam	13.15	309.1>281.1	7	4-100	0.9994
8	Chlordiazepoxide	12.61	300.1>227.1		4-100	0.9996
9	Clobazam	12.89	301.1>259.1		4-100	0.9996
10	Dextromethorphan	11.71	272.2>215.2		4-100	0.9996
11	Diazepam (w.IS)	13.44	285.1>193.1		4-100	0.9984
12	Flunitrazepam	12.70	314.1>268.1		4-100	0.9994
13	Flurazepam	11.86	388.2>315.0		4-100	0.9980
14	Lorazepam	12.92	321.0>275.0		4-100	0.9998
15	Mescaline	5.95	212.1>195.1		4-100	0.9984
16	Methylphenidate	8.69	234.15>84.1		4-100	0.9999
17	Midazolam	12.53	326.1>291.1		4-100	0.9992
18	Tramadol	8.41	264.2>58.0		4-100	0.9993
19	Cannabinol	13.86	311.2>222.9		4-100	0.9933
20	Anhydroecgonine methyl ester (w.IS)	3.06	182.1>91.1	8	4-100	0.9984
21	Estazolam (w.IS)	12.93	295.1>267.1	9	4-100	0.9975
22	Amitriptyline	12.99	278.1>233.0	10	4-100	0.9972
23	Desipramine	12.92	267.2>72.1		4-100	0.9941
24	Imipramine (w.IS)	12.85	281.2>86.1		4-100	0.9938
25	Trimipramine	13.03	295.2>100.1		4-100	0.9965
26	MDA (w.IS)	5.91	180.1>163.1	11	4-100	0.9961
27	MDEA (w.IS)	6.85	208.1>163.1	12	4-100	0.9966
28	3,4-Methylenedioxypyrovalerone	9.13	276.2>126.2	13	4-100	0.9960
29	Cathinone	4.30	150.1>117.1		4-100	0.9983
30	Fentanyl	11.28	337.3>188.2		4-100	0.9944
31	Ketamine	7.85	238.1>125.0		4-100	0.9943
32	LSD (Lysergic acid diethylamide)	9.92	324.2>223.1		4-100	0.9937
33	MDMA (w.IS)	6.11	194.1>163.1		4-100	0.9966
34	Mephedrone	6.95	178.1>145.1		4-100	0.9971
35	Methcathinone	4.69	164.1>131.1		4-100	0.9966
36	Sibutramine	13.11	280.2>125.1		4-100	0.9945
37	Methadone (w.IS)	12.98	310.2>265.2	14	4-100	0.9966
38	Methamphetamine (w.IS)	5.83	150.2>91.1	15	4-100	0.9980
39	Codeine (w.IS)	4.98	300.2>152.1	16	4-100	0.9989
40	Mitragynine (w.IS)	11.69	399.1>173.9	17	4-100	0.9978
41	Morphine (w.IS)	2.72	286.15>152.10	18	4-100	0.9952
42	Nalorphine	4.82	312.10>201.00	19	4-100	0.9901
43	Naloxone	4.98	328.15>212.10		4-100	0.9923
44	Naltrexone	5.60	342.15>270.15		4-100	0.9901
45	Nimetazepam	12.79	296.05>250.20		4-100	0.9940
46	Nitrazepam	12.50	282.10>236.10		4-100	0.9952
47	Nordiazepam (w.IS)	13.26	271.05>140.05		4-100	0.9964
48	Pentazocine	10.36	286.20>218.20		4-100	0.9964
49	Phencyclidine	10.71	244.20>91.05		4-100	0.9953
50	Norpseudoephedrine (w.IS)	4.21	151.95>134.05	20	4-100	0.9984
51	Nortriptyline (w.IS)	13.05	264.15>233.15	21	4-100	0.9913
52	Oxazepam (w.IS)	12.91	287.05>241.00	22	4-100	0.9932
53	Prazepam	13.80	325.10>271.05		4-100	0.9861
54	Sildenafil	12.67	475.20>58.05		4-100	0.9889
55	Temazepam	13.10	301.05>255.10		4-100	0.9976
56	Triazolam	13.09	343.05>308.20		4-100	0.9964
57	Oxycodone (w.IS)	5.32	316.15>241.15	23	4-100	0.9989
58	R-Pseudoephedrine (w.IS)	4.84	166.00>148.00	24	4-100	0.9976
59	Zolpidem (w.IS)	9.77	308.20>235.15	25	4-100	0.9991
60	Zaleplon	12.22	306.00>236.00		4-100	0.9962
61	Zopiclone (w.IS)	8.12	389.10>244.95	26	4-100	0.9968

Quantitative screening of spiked blood sample

A whole blood sample free of the listed targets was thawed from deep frozen storage and used as the blank (added IS, 4 ppb) and matrix to prepare spiked samples (added mixed targets at 10 ppb and IS 4 ppb) for determining the recovery and precision. A batch run was carried out on the CLAM-LC-MS/MS system, including solvent, blank (with 4 ppb IS), calibrants (4, 20 and 100 ppb, with 4 ppb IS), spike samples (10 ppb with 4 ppb IS) and QC sample (10 ppb with 4 ppb IS).

Recovery and precision of this screening workflow were evaluated with 10 ppb spiked whole blood sample by determining for five times (n=5) on the CLAM-LC-MS/MS system. A representative chromatogram is shown in Figure 4. The recovery was calculated by: R(%) = [Area $_{\text{in spiked sample}}$ / Area $_{\text{in neat}}$) X 100%. The results indicate that recoveries of 53 targets out of 61 and 23 deuterated ISs out of 26 are within 70%~130%. The compounds which recoveries were out of the range

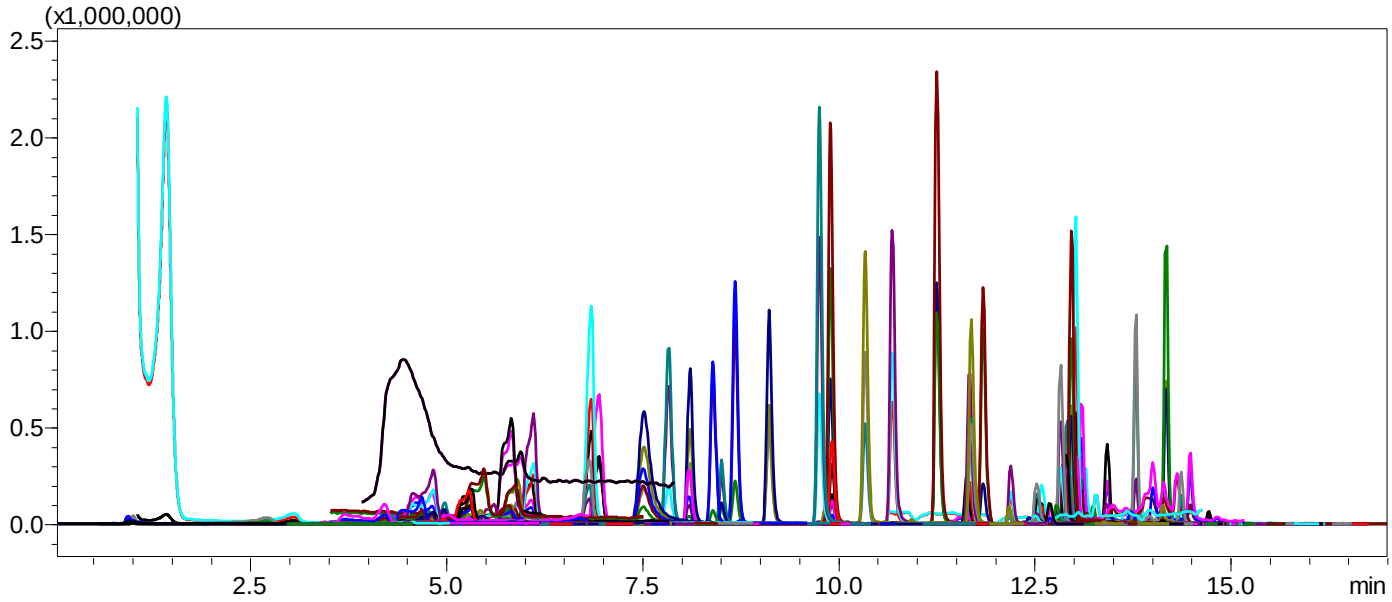
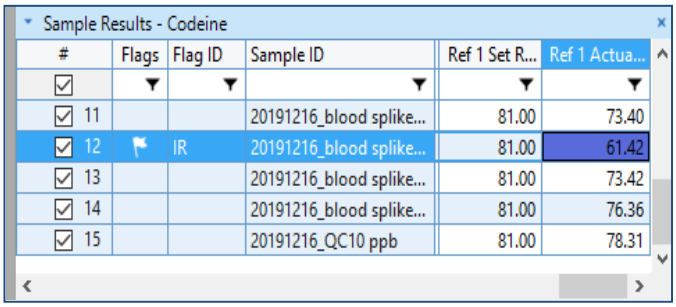


Figure 4: MS chromatogram of spike whole blood sample (targeted drugs at 10 ppb with IS at 4.0 ppb).

are 6-Acethyl Morphine (58.0%) and 6-Acethyl Morphine-d3 (32.3%); Carbamazepine (66.9%) and Carbamazepine-d10 (53.8%), as well as Midazolam (58.4%), Cannabinol (50.8%), Cathinone (41.3), Mephedrone (60.4), Methcathinone (45.2%), Nordiazepam (134.7%) and Morphine-d3 (152.2%). The precisions (RSD, n=5) of the above spiked samples are below 14.4% for the 61 targets and below 17.8% for the 26 internal standards.

It is worth to note that the Flag ID function of the LabSolutions Insight™ s/w was used in batch data analysis to alert large RT shift, unmatched ion ratio, poor linearity and accuracy etc., which provides a very efficient tool in quick and reliable checking of the results (Figure 5).


Sample Results - Codeine

#	Flags	Flag ID	Sample ID	Ref 1 Set R...	Ref 1 Actua...
11			20191216_blood splike...	81.00	73.40
12		IR	20191216_blood splike...	81.00	61.42
13			20191216_blood splike...	81.00	73.42
14			20191216_blood splike...	81.00	76.36
15			20191216_QC10 ppb	81.00	78.31

Figure 5: The “Flag ID” indicates the Ion Ratio of Codeine in a spike sample is out of the criteria (+/-15% absolute).

❑ Conclusion

A fully-automated platform consisting of CLAM-2000 and LC-MS/MS was used in establishing multi-target screening analysis for 61 toxicological compounds in whole blood samples. By using the method package Rapid Toxicology Screening, tedious method development work was avoided with only RT alignments and MRM optimization for some ISs. Co-injection with pure water after sample pre-treatment on CLAM module was found necessary to minimize the solvent effect in the subsequent LC elution. LabSolutions Insight s/w was used in data analysis. The Flag ID function of the s/w was used to alert RT shift, unmatched ion ratio etc. This work demonstrates that the systems and s/w used could be greatly helpful in establishment of high throughput screening analysis for a large number of targets in biological samples in toxicological research and investigation.

❑ References

1. Tiphaine Robin, Alan Barnes, Sylvain Dulaurent, Neil Loftus, Sigrid Baumgarten, Stéphane Moreau, Pierre Marquet, Souleiman El Balkhi and Franck Saint-Marcoux, Analytical and Bioanalytical Chemistry (2018) 410:5071–5083
2. Shimadzu Method Package - Rapid Toxicology Screening (Version 2), Shimadzu Corporation (Japan), https://www.shimadzu.com/an/news-events/2014/toxicology.html

❑ Acknowledgement

We sincerely thank Dr. Nat Tansrisawad, Udomsak Hoonwijit, Apinya Tubtimrattana, Boontariga Intawong, Samita Tanasarnsopaporn, Jakkapan Boonsritan from Department of Forensic Medicine, Chulalongkorn University, Mr. Chukkapong Comsup and Mr. Prapath Tienprateep from Bara Scientific Co for their valuable contribution in this work. – *Dr. Zhaoqi Zhan and Dr. Zhe Sun*

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