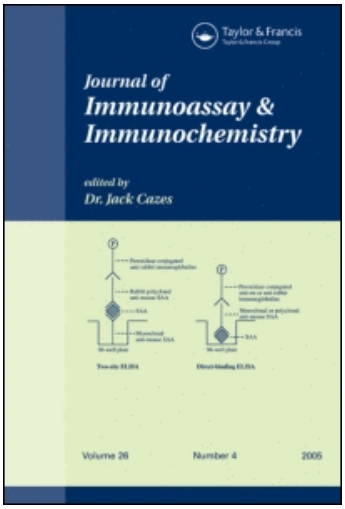

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Wendi Zhang[a]; Ping Su[a]; Yi Yang[a]; Zhenquan Guo[b]
[a] College of Science, Beijing University of Chemical Technology, Beijing, P. R. China [b] College of Life Sciences, Peking University, Beijing, P. R. China

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DEVELOPMENT OF A SENSITIVE BIOTIN–AVIDIN AMPLIFIED ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETERMINATION OF KETAMINE IN BIOLOGICAL SAMPLES

Wendi Zhang,[1] Ping Su,[1] Yi Yang,[1] and Zhenquan Guo[2]

[1]*College of Science, Beijing University of Chemical Technology, Beijing, P. R. China*
[2]*College of Life Sciences, Peking University, Beijing, P. R. China*

□ *An effective biotin–avidin amplified enzyme-linked immunosorbent assay (BA-ELISA) was developed to determine ketamine in biological samples. A conjugate of ketamine and ovalbumin (OVA) was used for immunization to produce polyclonal antibody. The conjugate of ketamine and bovine serum albumin (BSA) with polyclonal antibody was calculated to have an affinity constant (K_{aff}) of $3.30 \times 10^8 (mol/L)^{-1}$. The linear range of ketamine was 0.1–1000 μg/L with recoveries from 89.6% to 99.9% in spiked sample analysis. The detection limit of ketamine was 0.03 μg/L, which is more sensitive than that of the traditional ELISA. The results obtained by BA-ELISA agreed well with that of the traditional ELISA, with a correlation coefficient of 0.98.*

Keywords biotin–avidin, ELISA, HRP, immunoassay, ketamine, polyclonal antibody

INTRODUCTION

Ketamine [2-(-*o*-chlorophenyl)-2-(methylamino)-cyclohexanone] is a synthetic, sedative, nonbarbiturate, and fast-acting anesthetic. It can block the receptor of *N*-methyl-D-asparate (NMDA) and affect opioid, adrenergic, cholinergic, and serotonin receptors.[1] Ketamine is an administered anesthetic induction agent that provides prolonged relief of acute pain in surgical procedures. It is also used to treat some chronic pain conditions that are refractory to present therapies.[2] It was initially abused by medical personnel for its hallucinogenic effects and gradually became popular in Western and Asian countries. Sometimes ketamine is abused along with other drugs such as cocaine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), and benzodiazepines.[3,4] During the past decade, ketamine abuse has emerged as a public health concern. Ketamine is now

Address correspondence to Yi Yang, College of Science, Beijing University of Chemical Technology, Beijing 100029, P. R. China. E-mail: yangyi@mail.buct.edu.cn

considered a controlled substance. Pharmacokinetically, ketamine has a relatively short elimination half-life, with alpha-elimination phase lasting for only a few minutes, and beta-elimination phase lasting 2–3 hr.[1] At 72 hr after overdose injection, just about 2% of the dose is excreted in the urine as unchanged drug.[5] Therefore, a rapid, sensitive, and reliable method should be developed not only for the analysis of ketamine but also for the screening of suspected ketamine abusers.

Several methods have been reported for the determination of ketamine using a variety of techniques, including high-performance liquid chromatography with ultraviolet detection (HPLC-UV),[6–8] liquid chromatography–mass spectrometry (LC-MS),[9–12] gas chromatography-mass spectrometry (GC-MS),[13,14] and micellar electrokinetic chromatography (MEKC).[15,16] The samples containing ketamine are often complicated biological ones. A pretreatment step is often included before direct detection to provide proper concentration enhancement and matrix removal. The time-consuming sample cleanup process and the high price of equipment make these methods inapplicable to high-throughput laboratories for performing routine ketamine examination.

Enzyme-linked immunosorbent assay (ELISA) is an immunological technique with simplicity, sensitivity, and specificity that is widely used in various fields such as food safety,[17] public health,[18,19] and environmental protection.[20] It can be applied directly to the analysis of complex samples and can enable high throughput. As pointed out by the published articles,[21,22] this method is suitable for conducting large-scale screening of ketamine at lower levels in biological samples.

In this study, a high titer of polyclonal antibody against ketamine was obtained by immunizing rabbits. A biotin–avidin amplified enzyme-linked immunosorbent assay was established to determine ketamine using the biotinylated goat anti-rabbit immunoglobulin (Ig) G and streptavidin–horseradish peroxidase (HRP). The established method had a broad linear range with high sensitivity and specificity. The analytical potential of the immunoassay was demonstrated with satisfactory recoveries to determine ketamine in biological samples.

EXPERIMENTAL

Reagents and Chemicals

Ketamine, norketamine, and cocaine were obtained from China National Institute of the Control of Pharmaceutical and Biological Products. Morphine was purchased from Qinghai Pharmaceutical Factory (China). Potassium carbonate, potassium dihydrogen phosphate, and toluene were purchased from Beijing Chemical Plant (China). Bromoacetyl

bromide was purchased from Shangdong Jincheng Pharmaceuticals and Chemicals Co., Ltd (China). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIC), and 3,3′,5,5′-tetramethybenzidine (TMB) were purchased from Sigma Chemical Co. (USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, biotinylated goat anti-rabbit IgG, and streptavidin–HRP were obtained from Beijing Biotinge Biotech Co. (China).

Instrumentation

Microwell plates were obtained from Costar (USA), and a model 550 microplate reader (Tecan, Austria) was used to measure the optical densities of the ELISA results. A Cary 1E ultraviolet–visible (UV–Vis) spectrometer (Varian, USA) was used to measure the absorbance of the protein solution.

Buffers

All buffers and solutions were prepared with pure water (Milli-Q water purification system, Millipore, Billerica, MA, USA). The coating buffer was a 50 mol/L bicarbonate buffer (pH 9.6). A 10 mol/L phosphate buffered saline (PBS) was used as the assay buffer (pH 7.4). The wash buffer (PBST) was a PBS containing 0.05% Tween 20. The blocking solution was prepared by dissolving 1% glutin in PBS. The substrate solution was freshly prepared by mixing phosphate buffer (100 mol/L, pH 6.0), TMB (6 g/L in dimethyl sulfoxide [DMSO]), and H_2O_2(30%) at a volume ratio of 1000:10:1.5. The stopping solution was 2 mol/L H_2SO_4.

Preparation of Complete Antigen

The ketamine (100 mg) was dissolved in toluene (20 mL), and potassium carbonate (300 mg) was added subsequently. Bromoacetyl bromide (0.5 mL) was added to the preceding solution in droplets with constant stirring and in an atmosphere of nitrogen gas. The resulting mixture was continuously stirred for another 6 hr in nitrogen atmosphere at room temperature. The solvent was removed under vacuum and a yellow residue was obtained.

OVA solution was prepared by dissolving 50 mg OVA in 10 mL of 0.1 mol/L $NaHCO_3$. The residue was dissolved in the same solution and the OVA solution was added slowly in droplets with stirring. The reaction was performed for 24 hr and the solution obtained was dialyzed against 0.01 mol/L PBS at 4°C for 24 hr. Finally, the solution was lyophilized, yielding a white crystal. Then the crystal was stored at −20°C. The coupling ratio of ketamine-OVA conjugate was determined by the Coomassie brilliant blue method.[23] The structural formula of the complete antigen is shown in Figure 1.

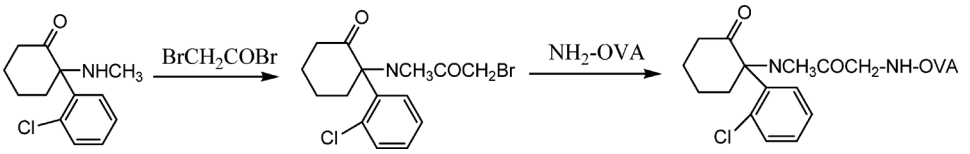

FIGURE 1 Synthesis of the complete antigen of ketamine.

The ketamine–BSA conjugate was prepared in the same way as for the ketamine–OVA.

Production and Purification of the Polyclonal Antibody

Three rabbits were immunized by subcutaneous injections. The first injection of ketamine–OVA at 1.0 mg was emulsified in complete Freund's adjuvant. Then it was followed by injections at a 12-day interval and half the quantity of immunogen was emulsified in incomplete Freund's adjuvant. Blood samples were taken 7 days after the final injection and tested by ELISA.

The antibody obtained was purified according to a modified caprylic acid-saturated ammonium sulfate (SAS) method.[24] The protein concentrations of the antiserum and the purified antibody solution were determined by ultraviolet spectroscopy and calculated based on the following equation: $C_{\text{protein}}(\text{g/L}) = 1.45A_{280\text{nm}} - 0.74A_{260\text{nm}}$. The purified antibody was stored at $-20°\text{C}$.

Titer and Affinity Constant

The titer of the purified antibody was determined by ELISA.[23] The ketamine–BSA conjugate was absorbed at 4°C in the wells of polystyrene microtiter strips. After being blocked with the blocking solution for 2 hr at 37°C, the wells were incubated with 10-fold serially diluted, purified antibodies for 1 hr at 37°C. Then 100 μL of the 0.20 mg/L HRP-conjugated goat anti-rabbit IgG was added and incubated for 1 hr at 37°C. The wells were rinsed and filled with substrate solution and the stopping solution was added after reacting for 15 min at room temperature. The absorbance of each well was read at 450 nm from the microplate reader. The affinity constant was measured with different concentrations of coating ketamine–BSA. Other steps were the same as described earlier.

BA-ELISA Procedure Used to Determine Ketamine

The wells of microtiter plates were coated with 2.00 mg/L ketamine–BSA and blocked in the same way as described in the preceding subsection. Standard solutions of one of the competitive compounds were added

together with purified antibody solution and incubated for 1 hr at 37°C. Then the wells were washed 3 times and 100 μL of the 0.20 mg/L biotinylated goat anti-rabbit IgG was added. The resulting wells containing the solutions were incubated for 1 hr at 37°C. Subsequently, the wells were washed again, but this was followed by the addition of 0.50 mg/L streptavidin–HRP and incubation for 40 min at 37°C. Then the same steps as in the preceding subsection to determine the titer of the purified antibody were repeated.

Application to the Biological Samples

The serum and urine samples obtained from valid volunteers were frozen and stored until analysis. The same competitive BA-ELISA procedure described earlier was used for quantitative detection of ketamine in human serum and urine samples with a known amount of ketamine.

RESULTS AND DISCUSSION

Preparation of Complete Antigen

As a small molecule, ketamine is not capable of initiating an immune response by itself. However, it can be used as a hapten to be conjugated to a large carrier protein to form a complete antigen. It is important to expose its characteristic group to the largest extent to select the conjugated position of the hapten. Bromoacetyl bromide was chosen as the binding carrier to activate the secondary amino group of ketamine, and the covalent attachment of the modified ketamine proceeded via the amino groups of lysine residues present in the OVA. The coupling ratios of ketamine–OVA conjugate and ketamine–BSA conjugate were measured as 1:20 and 1:21, respectively, by the Coomassie brillant blue method.

Determination of Titer and Affinity Constant

The concentration of the purified antibody was 11.54 g/L by UV measurement. Based on the definition of titer as the purified antibody required to bind 50% of a small, given amount of labeled antigen, the titer of the purified antibody was found to be 1:50,000.

The affinity constant (K_{aff}) represents the combined stability of the antigen–antibody complex, which is a primary parameter to estimate the quality of the anti-ketamine antibody. K_{aff} can be determined by ELISA based on the law of mass action.[25] The affinity constant of the anti-ketamine purified antibody with ketamine–BSA was 3.30×10^8 $(mol/L)^{-1}$, which was sufficient to be used in immunoassay method.

Optimization of Immunoassays

Different parameters affecting the sensitivity of assay were optimized experimentally. The optimal concentrations of the coated antigen and the purified antibody play major roles in the determination of ketamine. Figure 2 shows the dilution curves of anti-ketamine antibody reacting with ketamine–BSA at different coated antigen concentrations in BA-ELISA. According to the shape of the sigmoid curves, 2.00 mg/L was chosen as the optimal coating concentration for ketamine–BSA. The optimal dilution of purified anti-ketamine antibody for the BA-ELISA used was determined to be 1:5000 (2.29 mg/L) from the serial dilutions of anti-ketamine purified antibody curves at different coated antigen concentrations.

A dilution series of biotin and and a dilution series of streptavidin–HRP were prepared to obtain optimal BA-ELISA assay concentrations. The biotin dilution started at 1:5000 (0.20 mg/L), whereas streptavidin–HRP started at 1:500 (1 mg/L). As shown in Figure 3, the optimal concentrations of biotin and streptavidin–HRP were found to be 0.20 mg/L and 0.50 mg/L, respectively. The reaction time of biotin and streptavidin was also investigated in order to improve the sensitivity. The reaction times of 10, 20, 30, 40, and 50 min were tested. As shown in Figure 4, the optical density was low at the beginning of the reaction, but became high and remained

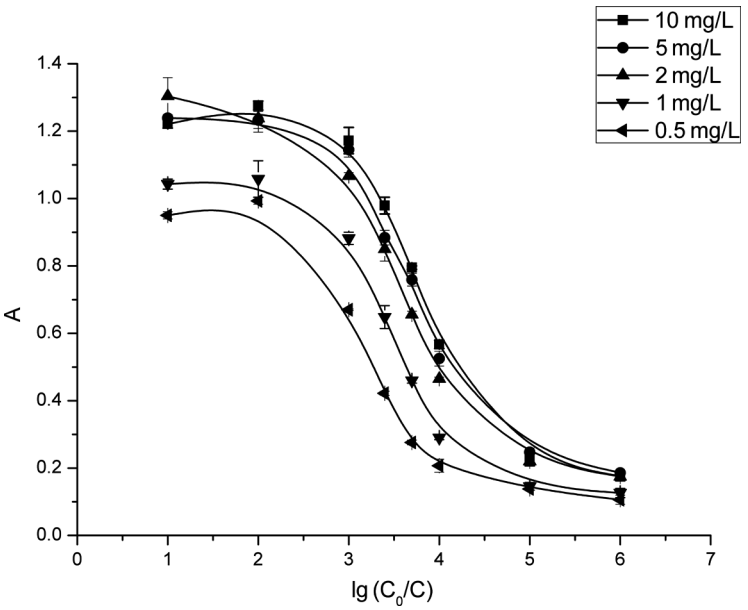

FIGURE 2 The dilution curves of anti-ketamine antibody reacting with ketamine–BSA at different coated antigen concentrations (0.5, 1, 2, 5, and 10 mg/L) in BA-ELISA. Data points are the average plus/minus one standard deviation ($n = 5$).

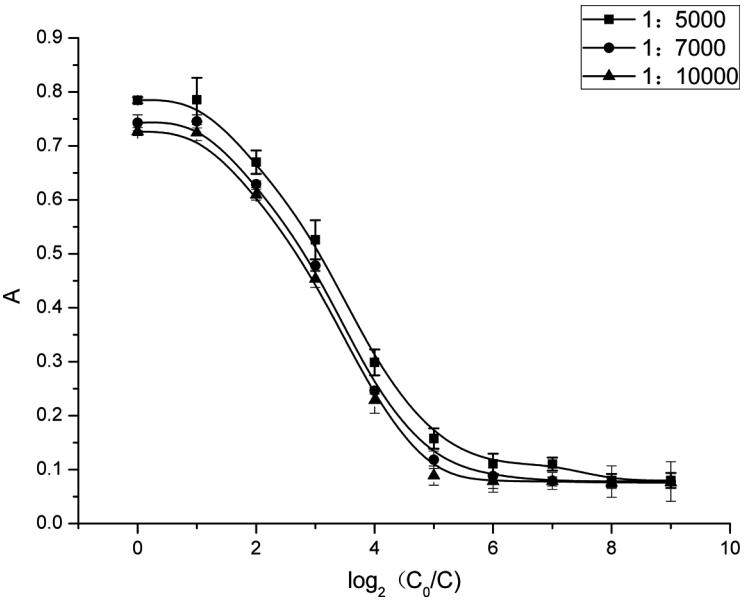

FIGURE 3 Two-time serial dilution curve of streptavidin–HRP in ELISA with primary concentration of 1 mg/L at different dilutions of biotin–IgG. The biotin–IgG dilution started at 1:5000 (0.20 mg/L). Data points are the average plus/minus one standard deviation ($n = 5$).

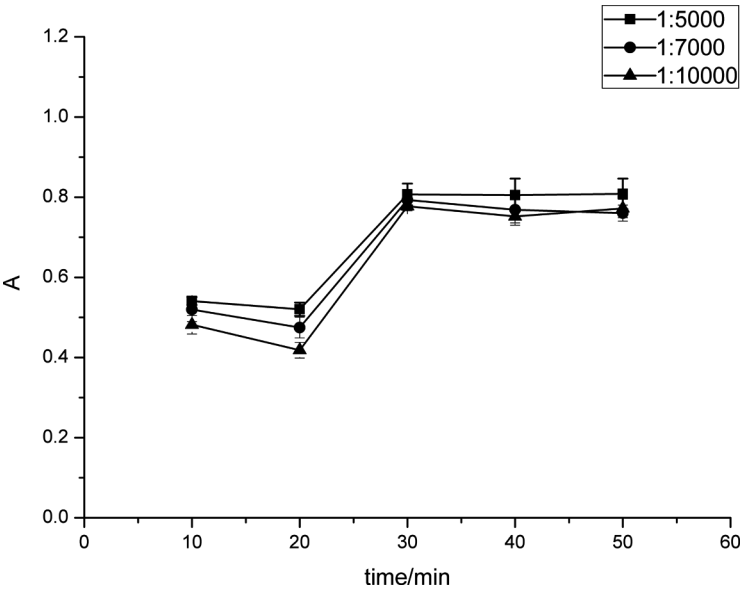

FIGURE 4 Time curves of serially diluted biotin–IgG reacting with streptavidin–HRP in ELISA. The biotin–IgG dilution started at 1:5000 (0.20 mg/L). Data points are the average plus/minus one standard deviation ($n = 5$).

stable after 30 min. As a result, 40 min was selected as the suitable reaction time of biotin and streptavidin–HRP.

Analytical Performance of BA-ELISA

Calibration

The calibration was obtained using 2.00 mg/L ketamine–BSA conjugate and 2.29 mg/L purified antibody. The detection limit was 0.03 μg/L, defined as blank plus 3 times standard deviation of the blank. The linear range was between 0.1 and 1000 μg/L. The equation of the calibration curve was represented as $\text{Absorbance} = 0.05685 + 0.07865[\log(C_0/C)]$ ($R^2 = 0.9889$, $n = 5$). The intra-assay and interassay coefficients of variation were 6.02% and 8.11%, respectively.

Specificity

Specificity in assay can be defined as the measurable response for only the target analyte. Cross-reactivity is a measurement of antibody response to substances that have a structure similar to that of the analyte. When the structurally related compounds are present in the sample, they can influence the results. Ketamine, norketamine, morphine, and cocaine were investigated. Table 1 showed the results of cross-reactivities of different compounds. According to the 50% replacement method,[26] the cross-reactivity of norketamine was 35.13%, while those of the other two compounds were less than 2%. The low cross-reactivity of antibody for norketamine is understandable because norketamine is a metabolite of ketamine and has a structure similar to ketamine's. Therefore, the BA-ELISA developed for ketamine was highly specific.

Application to the Biological Samples

The ultimate goal of this study was to develop a simple method for the analysis of ketamine in biological samples. Applicability of the developed method to real samples was tested by the analysis of serum and urine samples obtained from healthy subjects. For the serum samples, a 1:10

TABLE 1 Cross-Reactivities of Ketamine, Norketamine, Morphine, and Cocaine

Cross-Reactants	Cross-Reactivity (%)
Ketamine	100.00
Norketamine	35.13
Morphine	1.09
Cocaine	0.48

TABLE 2 Recoveries of Ketamine in the Serum and in Urine Samples

	Recovery (%)[a]	
Ketamine(μg/L)	Serum Sample	Urine Sample
0.9	93.22 ± 8.97	99.22 ± 6.84
9	93.34 ± 8.97	89.55 ± 1.67
90	99.87 ± 6.75	95.20 ± 9.76

[a]The data were expressed as mean ±SD of 10 experiments.

dilution with 0.01 mol/L PBS, pH 7.4, was necessary prior to being added to the wells to obtain quantitative recoveries. The urine samples were also diluted 10-fold with PBS in order to eliminate matrix effect. The recovery results, which indicate the average recoveries of spiked human serum and those of spiked human urine as shown in Table 2, range from 93.22% to 99.87% and 89.55% to 99.22%, respectively. The ketamine-free samples (i.e., nonspiked samples) were also included in the analysis as negative controls.

TABLE 3 Analyses by BA-ELISA and ELISA of Serum Spiked with Ketamine at 0, 0.9, 9, and 90 μg/L

Number	Spiked Concentration (μg/L)	Detection of ELISA Method (μg/L)	Detection of BA-ELISA Method (μg/L)
1	0	N.D.	N.D.
2	0	N.D.	N.D.
3	0	N.D.	N.D.
4	0	N.D.	N.D.
5	0	N.D.	N.D.
6	0.9	0.79	0.81
7	0.9	0.84	0.89
8	0.9	0.92	0.94
9	0.9	0.77	0.80
10	0.9	0.80	0.82
11	9.0	9.90	8.73
12	9.0	9.81	8.38
13	9.0	10.17	9.52
14	9.0	9.08	8.19
15	9.0	9.01	7.90
16	90	84.85	89.10
17	90	84.61	88.22
18	90	81.89	80.70
19	90	87.32	93.06
20	90	89.15	97.91

Note. N.D. = not determined.

TABLE 4 Analyses by BA-ELISA and ELISA of Urine Spiked with Ketamine at 0, 0.9, 9, and 90 μg/L

Number	Spiked Concentration (μg/L)	Detection of ELISA Method (μg/L)	Detection of BA-ELISA Method (μg/L)
1	0	N.D.	N.D.
2	0	N.D.	N.D.
3	0	N.D.	N.D.
4	0	N.D.	N.D.
5	0	N.D	N.D.
6	0.9	0.78	0.86
7	0.9	0.96	0.89
8	0.9	0.80	0.81
9	0.9	0.72	0.84
10	0.9	0.77	0.79
11	9.0	8.55	7.98
12	9.0	10.17	8.04
13	9.0	9.63	8.38
14	9.0	10.17	8.64
15	9.0	9.08	8.21
16	90	99.68	98.23
17	90	98.17	84.78
18	90	97.91	83.48
19	90	84.90	80.25
20	90	98.10	83.15

Note. N.D. = not determined.

Comparison with ELISA Analysis

ELISA was applied as a reference method to determine ketamine. The linear range was between 1 and 10,000 μg/L. The regression equation of the curve was represented as: Absorbance $= 0.1556 + 0.0656[\log(C_0/C)]$ ($R^2 = 0.9926$, $n = 5$). The detection limit was 0.58 μg/L.

Spiked biological samples were analyzed for ketamine, both by the BA-ELISA and by the ELISA. The results are shown in Tables 3 and 4. Correlation between the BA-ELISA results and the ELISA results showed a good linear relationship, with $R^2 = 0.98$. Compared with the ELISA, the BA-ELISA has a lower detection limit and can be applied to the determination of ketamine at lower levels.

CONCLUSIONS

A biotin–avidin amplified enzyme-linked immunosorbent assay (BA-ELISA) has been developed for the determination of ketamine in biological samples with acceptable precision and accuracy. It is more sensitive than ELISA, with a detection limit of 0.03 μg/L. The established method was compared with the traditional ELISA. The results show that the two

methods agree with each other very well in the assay of serum and urine samples. The presented BA-ELISA method can be considered as a rapid and cost-effective technique to examine ketamine with a reasonable sensitivity. It is suitable for the analysis of ketamine in biological samples, especially for high-throughput screening.

REFERENCES

1. Smith, K.M.; Larive, L.L.; Romanelli, F. Club drugs: methylenedioxymethamphetamine, flunitrazepam, ketamine hydrochloride, and gamma-hydroxybutyrate. Am. J. Health Syst. Pharm. **2002**, *59*(11), 1067–1076.
2. Lin, T.C.; Wong, C.S.; Chen, F.C.; Lin, S.Y.; Ho, S.T. Long-term epidural ketamine, morphine and bupivacaine attenuate reflex sympathetic dystrophy neuralgia. Can. J. Anaesth. **1998**, *45*(2), 175–177.
3. Chen, C.Y.; Lee, M.R.; Cheng, F.C.; Wu, G.J. Determination of ketamine and metabolites in urine by liquid chromatography–mass spectrometry. Talanta **2007**, *72*, 1217–1222.
4. Gentili, S.; Cornetta, M.; Macchia, T. Rapid screening procedure based on headspace solid-phase microextraction and gas chromatography–mass spectrometry for the detection of many recreational drugs in hair. J. Chromatogr. B **2004**, *801*(2), 289–296.
5. Kim, E.M.; Lee, J.S.; Choi, S.K.; Lim, M.A.; Chung H.S. Analysis of ketamine and norketamine in urine by automatic solid-phase extraction (SPE) and positive ion chemical ionization–gas chromatography–mass spectrometry (PCI–GC–MS). Forens. Sci. Int. **2007**, *217*, 1–6.
6. Niedorf, F.; Bohr, H.H.; Kietzmann, M. Simultaneous determination of ketamine and xylazine in canine plasma by liquid chromatography with ultraviolet absorbance detection. J. Chromatogr. B **2003**, *791*, 421–426.
7. Gross, A.S.; Nicolay, A.; Eschalier, A. Simultaneous analysis of ketamine and bupivacaine in plasma by high-performance liquid chromatography. J. Chromatogr. B **1999**, *728*, 107–115.
8. Fan, Y.; Feng, Y.Q.; Da, S.L.; Gao, X.P. In-tube solid-phase microextraction with poly(methacrylic acid-ethylene glycol dimethacrylate) Monolithic capillary for direct high-performance liquid chromatographic determination of ketamine in urine samples. Analyst **2004**, *129*, 1065–1069.
9. Rodriguez Rosas, M.E.; Patel, S.; Wainer, I.W. Determination of the enantiomers of ketamine and norketamine in human plasma by enantioselective liquid chromatography–mass spectrometry. J. Chromatogr. B **2003**, *794*, 99–108.
10. Cheng, J.Y.K.; Mok, V.K.K. Rapid determination of ketamine in urine by liquid chromatography–tandem mass spectrometry for a high throughput laboratory. Forens. Sci. Int. **2004**, *142*, 9–15.
11. Wang, K.C.; Shih, T.S.; Cheng, S.G. Use of SPE and LC/TIS/MS/MS for rapid detection and quantitation of ketamine and its metabolite, norketamine, in urine. Forens. Sci. Int. **2005**, *147*, 81–88.
12. Brown, S.D.; Rhodes, D.J.; Pritchard, B.J. A validated SPME-GC-MS method for simultaneous quantification of club drugs in human urine. Forens. Sci. Int. **2007**, *171*, 142–150.
13. Cheng, P.S.; Fu, C.Y.; Lee, C.H.; Liu, C.; Chien, C.S. GC–MS Quantification of ketamine, norketamine, and dehydronorketamine in urine specimens and comparative study using ELISA as the preliminary test methodology. J. Chromatogr. B **2007**, *852*, 443–449.
14. Chou, S.L.; Yang, M.H.; Ling, Y.C.; Giang, Y.S. Gas chromatography-isotope dilution mass spectrometry preceded by liquid–liquid extraction and chemical derivatization for the determination of ketamine and norketamine in urine. J. Chromatogr. B **2004**, *799*, 37–50.
15. Lin, Y.H.; Lee, M.R.; Lee, R.J.; Ko, W.K.; Wu, S.M. Hair analysis for methamphetamine, ketamine, morphine and codeine by cation-selective exhaustive injection and sweeping micellar electrokinetic chromatography. J. Chromatogr. A **2007**, *1145*, 234–240.
16. Lin, Y.H.; Li, J.H.; Ko, W.K.; Wu, S.M. Direct and sensitive analysis of methamphetamine, ketamine, morphine and codeine in human urine by cation-selective exhaustive injection and sweeping micellar electrokinetic chromatography. J. Chromatogr. A **2006**, *1130*, 281–286.

17. Jeon, M.; Kim, J.; Paeng, K.J.; Park, S.W.; Paeng, I.R. Biotin–avidin mediated competitive enzyme-linked immunosorbent assay to detect residues of tetracyclines in milk. Microchem. J. **2008**, *88*, 26–31.
18. Aroonrerk, N.; Suksamrarn, A.; Kirtikara, K. A sensitive direct ELISA for detection of prostaglandin E2. J. Immunoassay Immunochem. **2007**, *28*, 319–330.
19. Khatun, S.; Nara, S.; Tripathi, V.; Rangari, K.; Chaube, S.K.; Kariya, K.P.; Kumar, S.; Shrivastav, T.G. Development of ELISA for measurement of progesterone employing 17-a-OH-P-HRP as enzyme label. J. Immunoassay Immunochem. **2009**, *30*, 186–196.
20. Liu, Y.H.; Jin, M.J.; Gui, W.J.; Cheng, J.L.; Guo, Y.R.; Zhu, G.N. Hapten design and indirect competitive immunoassay for parathion determination: correlation with molecular modeling and principal component analysis. Anal. Chim. Acta **2007**, *591*, 173–182.
21. Huang, M.H.; Wu, M.Y.; Wu, C.H.; Tsai, J.L.; Lee, H.H.; Liu, R.H. Performance characteristics of ELISAs for monitoring ketamine exposure. Clin. Chim. Acta **2007**, *379*, 59–65.
22. Tan, M.E.C.; Moy, H.Y.; Lui, C.P.; Lee, T.K. Evaluation of an ELISA test kit in the screening of ketamine in urine. Forens. Sci. Int. **2003**, *136*, 305.
23. Zhao, M.P.; Li, Y.Z.; Guo, Z.Q.; Zhang, X.X.; Chang, W.B. A new competitive enzyme-linked immunosorbent assay (ELISA) for determination of estrogenic bisphenols. Talanta **2002**, *57*, 1205–1210.
24. Tang, D.; Wang, Y.C.; Chang, W.B.; Ci, Y.X.; Guo, Z.Q. Time-resolved fluorescence immunoassay of estradiol in serum. Chin. J. Anal. Chem. **1999**, *27*, 899–903.
25. Beatty, J.D.; Beatty, B.G.; Vlahos, W.G. Measurement of monoclonal antibody affinity by non competitive enzyme immunoassay. J. Immunol. Methods **1987**, *24*, 173–179.
26. Pratt, J.J. Steroid immunoassay in clinical chemistry. Clin. Chem. **1978**, *24*, 1869–1890.