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# Performance characteristics of ELISAs for monitoring ketamine exposure

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**Abstract**

*Background:* Growing misuse of ketamine necessitates the development of high throughput testing approaches. Two commercial enzyme-linked immunosorbent assays (ELISA) for ketamine have recently become available and were adapted for this development.
*Method:* The newly available ketamine ELISA reagents were examined to better understand their cross-reacting, calibration and other performance characteristics. ELISA apparent analyte concentrations were also correlated against ketamine concentrations as determined by GC-MS to examine the relationship between these 2 parameters.
*Result:* Both adapting ketamine as the targeted analyte, reagent from International Diagnostic Systems (IDS) also responded very significantly to the metabolites of ketamine (norketamine and dehydronorketamine), while the NEOGEN reagent responded very specifically to ketamine.
*Conclusion:* NEOGEN ELISA test data exhibit better correlation with the ketamine concentration as determined by GC-MS. It can be more reliably used as the preliminary test method in the 2-step approach now routinely adapted in workplace drug testing programs. Using 100 ng/ml ketamine as the GC-MS cutoff, the corresponding ELISA cutoff value is approximately 110–120 ng/ml. With significantly higher responses to ketamine metabolites, IDS reagent can detect specimens with much lower ketamine/metabolites concentrations and can better meet other testing requirements.


*Keywords:* Ketamine; Enzyme-linked immunosorbent assay; Gas chromatography–mass spectrometry

## 1. Introduction

Synthesized in 1962 [1] and marketed as an anesthetic agent for human and animal use in 1970s [2], ketamine (K) is now widely abused in Western and Asian countries [3]. It is reportedly the fastest growing drug of abuse in Taiwan [4]. This abuse trend has created a need for toxicological laboratories to better understand K's metabolic and excretion characteristics [5–8] and to develop methods for the analysis of K and its metabolites [5–16].

As pointed out by 2 articles published in 2004 [11,12], there was no commercially available immunoassay for K and its metabolites until very recently. To the best of our knowledge, reports related to immunoassays for ketamine are limited to one abstract presented in 2003 [10], 2 articles published in 2005 involving the use of a commercial ELISA kit to monitor urinary excretion of K and norketamine (NK) from human [10] and monkeys [8], and a poster presentation on the development of a homogeneous assay [17]. With this in mind, this study was designed to evaluate the performance characteristics of the only 2 micro-plate well ELISA kits currently available from commercial sources, with emphasis on their cross reactivity toward the metabolites, NK and dehydronorketamine (DHNK), which often present at higher concentration than K.

Further emphasis was placed on the correlation of the *apparent* analyte concentration as determined by ELISA against true K concentration (as determined by GC-MS). The resulting correlation data were utilized to estimate ELISA apparent analyte concentrations that correspond to specific K GC-MS concentrations. When this correlation is established, a high throughput 2-step approach (as now routinely applied to testing the 5 drug categories regulated by U.S. Department of Health and Human Services) can then be developed [18].

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## 2. Materials and methods

### 2.1. Reagents, standard and specimens

Ketamine ELISA kits were kindly provided (free of charge) by NEOGEN Corp. (Lexington, KY) and International Diagnostic Systems Corp. (St. Joseph, MI). K, NK, K-$d_4$, and NK-$d_4$ (in 1 or 0.1 mg/ml methanol solutions) were from Cerilliant (Austin, TX). Dehydronorketamine (DHNK) was custom-synthesized by Prof. Ta-Jung Lu of National Chung-Hsing University (Taichung, Taiwan). Derivatization reagent, pentafluorobenzoyl chloride, was from Alfa Aesar (Lancs, Germany). Extraction solvents, triethylamine and cyclohexane, were from Ferak (Berlin, Germany) and Yak Uri Pure Chemical Co. (Osaka, Japan), respectively.

Clinical urine specimens were collected from patients seeking treatment. Patients were informed of the utilization of these specimens for the development of analytical methodology and signed the consent forms, following the regulation established by Kaohsiung Medical University (Kaohsiung, Taiwan). All specimens were kept frozen or at 4 °C until analysis.

### 2.2. Micro-plate well ELISA

Assay procedures provided by respective ELISA kit manufactures were followed and briefly outlined in Fig. 1. Dose-response calibrations were established using a series of standard solutions with predetermined concentrations. These standards were prepared by diluting the standards provided by the ELISA reagent manufacturers or standard compounds from other sources. Buffer (provided by ELISA reagent manufacturers) or drug-free urine were used as the matrix for the preparation of standard solutions.

For cross-reactivity studies, standards were diluted to preset concentrations and tested. The observed concentrations were then divided by the targeted concentrations to derive the test compounds' cross-reacting characteristics toward the ELISA kits examined. For the assay of clinical specimens, all specimens were diluted with drug-free urine by a predetermined factor.

### 2.3. Liquid–liquid extraction and derivatization [13]

One-ml aliquots of standards and controls (both prepared in drug-free urine) and test specimens were mixed with 1-ml $Na_2CO_3$/$NaHCO_3$ buffer (pH 9.0) and extracted with 4 ml triethylamine/cyclohexane mixture (3:1, v/v). After 10 min mechanical shaking, the mixture was centrifuged at 3000 rpm for 5 min. The top layer was transferred to a clean tube and dried with a slow stream of nitrogen at 50 °C. The residue was dissolved in 50 µl derivatizing reagent (pentafluorobenzoyl chloride) and incubated at 105 °C for 1 h. The derivatization product was cooled and evaporated to dryness under a slow stream of nitrogen at 50 °C. The residue was reconstituted with ethyl acetate immediately prior to GC-MS analysis. Typically, 200 µl of ethyl acetate was used for reconstitution and 1 µl was injected.

| Step | Neogen | IDS |
|---|---|---|
| Load sample/calibrators | 20 µL | 20 µL |
| ↓ | | |
| Add drug-enzyme conjugate | 180 µL | 100 µL |
| ↓ | | |
| Incubation | 45 min | 30 min |
| ↓ | | |
| Add wash buffer | 300 µL | 300 µL |
| ↓ | | |
| Add substrate | 150 µL (K-Blue) | 150 µL |
| ↓ | | |
| Add stop solution | 50 µL (1-N HCl) | 150 µL (1-N $H_2SO_4$) |
| ↓ | | |
| Read plate | 450 nm | 450 nm |

Fig. 1. Schematic presentation of IDS and NEOGEN ketamine ELISA test procedures.

### 2.4. Gas chromatography–mass spectrometry analysis

An Agilent 6890 gas chromatograph-5975N mass selective detector (GC-MS) system was used for this study. The gas chromatograph was equipped with 30-m Hewlett-Packard (Andover, MA) 5MS fused silica capillary column (0.25-mm ID; 0.25-µm film thickness). The injector and interface temperature were maintained at 260 and 280 °C, respectively. The oven temperature of the GC was set at 150 °C for 1 min, then increased to 250 °C at 12.5 °C/min and held for 1 min, then ramped at a rate of 10 °C/min to 280 °C and held for 2 min. At last, the temperature was increased to 300 °C at the rate of 20 °C/min and held for 2 min. (The detector was turned off at the last programming step.) The following injection parameters were adapted: sample size, 1 µL; injection mode, splitless; injector purge-off duration, 1 min.

Full-scan mass spectra were collected at the $m/z$ 50–500 range. Typical mass spectra of the analytes and internal standards are shown in Fig. 2. Based on ion intensity data of the full-scan mass spectra, ion pairs potentially can be used for designating the analyte/internal standard were evaluated and the following ion pairs were found most suitable [19]: $m/z$ 360, 152, 368 and 364, 156, 372 (for K and K-$d_4$); and $m/z$ 354, 382, 312 and 358, 386, 316 (for NK and NK-$d_4$). $m/z$ 312, 380, 138 were selected for designating DHNK. The intensity cross-contribution characteristics of ion pairs selected for the K/K-$d_4$ and the NK/NK-$d_4$ systems are shown in Table 1. The first ion listed for each compound was adapted for quantitation using 6-point (0, 40, 100, 400, 1000, 2000 ng/ml for K and NK and 0, 100, 400, 1000, 10000, 20000 ng/ml for DHNK) calibration curves. NK-$d_4$ was also adapted as the IS for the quantitation of DHNK.

## 3. Results and discussion

### 3.1. Characteristics of calibration curves

Calibration curve characteristics of the IDS and NEOGEN ELISA reagents were examined at the very beginning of this study. Since K was the targeted analyte for both reagents, dose-response curves were established using K as the target compound. Shown on the left of Fig. 3 are the curves derived from the reagents provided by IDS (A) and NEOGEN (B). The curve resulting from the IDS reagent appears to show a wider "linear" range. This characteristic becomes more apparent when the concentration axis is converted into log-scale as shown on the right of Fig. 3. Note that, within the same 50–1000 ng/ml concentration range, the slope of "A" line is steeper and the "B/B0" values change from approximately 70% to 30%, while that for the "B" line are from approximately 32% to 10%. It should also be noted, however, these calibration curves were established using standard solutions containing K alone, cross reactivity toward NK and DHNK was not taken into consideration.

### 3.2. Cross-reacting characteristics

Reagent package inserts provided by the manufacturers include comprehensive lists of compounds toward which no cross-reactivity was observed. These inserts also show differences in their cross-reactivity toward NK (see Table 2). Since most K-containing specimen also include significant amount of DHNK, we have also determined these reagents' cross-reactivity toward this compound as well as NK at several