Comparison among single-phase test, automated screening method and GC/MS for the traceability of ketamine in urine

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ABSTRACT

Background: The use of ketamine, for non-medical purpose, results widespread also in Italy. This drug is not searched by institutional centres, charged of the responsibility to realise the execution of toxicological analysis based on the article 187 of The New Italian Highway Code. We evaluated the reliability of the single-phase test comparing it with an automated screening method and a gas chromatography-mass spectrometry to search the presence of ketamine on casualty patients involved in car accidents in Rome.
Methods: The screening analyses were performed by a single-phase test (with a cut-off settled at 1000 ng/ml), and an automated immune enzymatic assay (cut-off settled at 330 ng/ml). The confirmation tests have been realised by gas chromatography-mass spectrometry.
Results: The single-phase test highlighted ten positive samples out of 294. The automated instrument confirmed only six out of ten previous positive samples, meanwhile the instrument found further four positive samples, considered negative by the single-phase test. The presence of ketamine is confirmed by gas chromatography-mass spectrometry only in seven samples out of fourteen resulted positives from both screening analysis. Three samples out of seven confirmed by gas chromatography-mass spectrometry were positive only to ketamine.
Conclusion: Comparing the two screening methods, we find a high difference of sensitivity and specificity.
The different results between screening methods detect the dissimilar reliable of tests.
The automation and the standardisation of methodology and analytical procedures is essential to guarantee the reliability of toxicological screening tests, especially to medico-legal significance. This results highlight the absolutely necessity of the execution of the confirmation test, successively to screening analysis.

Keywords: ketamine, Rome, car accidents, urine

INTRODUCTION

Ketamine is an arylcycloalkylamine, which is an amine bound to a benzene structure and a non-benzene structure defined as alicyclic. Ketamine is a synthetic molecule; it was created in a laboratory in 1962 and patented in Belgium in 1963 for clinical use as an anaesthetic and analgesic. In fact, in the 1960s a new branch of anaesthesia called *dissociative anaesthesia* was formed due to the ability to cause an 'out of body experience', inducing a loss of response to pain stimuli, as well as to the surrounding environment. First, phencyclidine and then ketamine was used with exactly this aim. The state of anaesthesia produced by ketamine was first described in terms of a functional and electrophysiological disassociation between the thalamo-neocortical system and the limbic system [1]. Ketamine's primary effect is upon the brain's thalamo neo-cortical projection, where the neurons of the cerebral cortex and the thalamus are selectively inhibited, and at the same time, those of the limbic system, including the hippocampus are stimulated. This creates a situation of 'functional disorganisation' of the brain, which induces anaesthesia, altered emotional state and hallucinations. As with every other type of narcotic substance, its effect in qualitative terms and its duration of action is strictly determined by the quantity taken and the method of ingestion. Ketamine is capable of generating various different states of altered consciousness, and it is exactly for this reason that it is used in the quest for new sensory experiences and recreationally [2]. The psychedelic quality of ketamine is very different to that of other hallucinogens like LSD, mescaline and psilocybin [3]. This drug is its capacity to produce, in some consumers, if taken at high doses, a *near death* experience [4,5] defined in slang as "K hole". The anaesthetic and amnesic effects of this substance is often used with the aim of committing sexual violence. The increase of road accidents due to driving under the influence of alcohol or drugs is currently an increasing problem [6,7]. Many recent studies show the negative effect of Ketamine on driving performance [7,8]. In Italy, the New Highway Code has established under articles 186 and 186bis that a driving under the influence of alcohol is punishable by law; similarly, under article 187 a driver found to be in an altered state of consciousness due to taking either narcotic or psychotropic substances may also be punished by the law. A positive test involves a penalty and an administrative sanction. Ascertaining whether or not a driver is under the influence of drugs or alcohol may initially be done using qualitative non-invasive methods at the roadside, and following that through taking samples of body fluids in hospital. As a matter of routine, samples are screened for cannabinoid, cocaine and opiate metabolites, as well as amphetamine/methamphetamine, MDMA, benzodiazepine, buprenorphine and methadone; however, these are not the only substances used today. Indeed, according to Italian epidemiological data, the use of ketamine, in particular, is an emerging problem [9,10]. Ketamine is not included in the list of substances to analyse, based on the Guidelines 2012 Gruppo Tossicologi Forensi Italiani (GTFI). The law gives to public hospitals the responsibility to realise the execution of toxicological analysis of medico-legal significance. The research of principal drugs' catabolites on urine by screening tests [11] is followed by confirmation test [12]. In our previous works [13,14,15] we have already remarked the presence of ketamine on casualty patients involved in car accidents in Rome [16]. The assumption of ketamine by these patients occurred for voluptuary purpose: ketamine was administered to anyone of them for medical purpose. The aim of this work is to value the reliability of two screening methods to research the presence of ketamine. The results obtained by screening methods have been compared each other. To estimate the sensitivity and specificity of the two methods, the samples resulted positive by screening tests have been confirmed by gas chromatography-mass spectrometry (GC / MS).

METHODS

Two screening methods have been used: a single-phase test and an automated method. The single-phase test is a one-step lateral flow chromatographic immunoassay based on the principle of competition for limited antibody binding sites between the drug or drug metabolites in the sample and a drug-protein conjugate immobilised on a porous membrane support. This test uses particles of monoclonal rat antibodies, which correspond to the protein conjugates of ketamine with the aim of showing 2–4% of the drug excreted in an unaltered form with a cut-off of 1000 ng/ml being sufficient to reveal it. The ketamine present in the sample competes with its relative conjugate for the same binding sites on the antibody. One drop of urine has dispensed on membrane in the spot for the samples. The matrix moves along the support by capillarity and reaches the region of reaction where are conjugated the antibodies. Urine moves along the membrane by way of capillarity. If the concentration of substance is less than the cut off, it will not be able to saturate all the binding sites of its specific antibodies which react, therefore, with the conjugate protein. The formation of a coloured line visible in the reactive area indicates the absence of competition due to a concentration of ketamine in the sample lower than the cut off. If the drug is present in concentrations that are higher than the cut off, then all the binding sites of the antibody are saturated and consequently, no coloured line may be seen due to competition between the two substances for the same antibody. To check that the test has been successful, a band will appear in the

control zone containing goat antibodies, which indicates that the correct amount of sample has been used and that the sample has moved along the membrane [4]. The other screening method is based on the automated immune enzymatic assay (cut-off settled at 330 ng/ml), uses a highly specific monoclonal antibody that can detect both Ketamine and its major metabolite Norketamine in human urine. The assay is based on competition between drug labelled with glucose-6-phosphate dehydrogenase (G6PDH) and free drug from urine sample for a fixed amount of antibody binding sites. In the absence of free drug from the sample, the specific antibody binds the enzyme labelled drug causing a decrease in enzyme activity. In the presence of free drug from the sample, the drug occupies the antibody binding sites, and leaves the drug labelled G6PDH free and active. This phenomenon creates a direct relationship between the drug concentration in urine and enzyme activity. The enzyme activity is determined spectrophotometrically at 340 nm by measuring its ability to convert NAD to NADH. For this analysis, we have used instrument Thermo Scientific Indiko. The confirmation tests carried out in the present work [17], has been realised with gas chromatography-mass spectrometry (GC / MS) instrument Agilent 7890A with helium as the carrier gas. GC / MS is a technique that combines the possibility of separation of gas chromatography with mass spectrometry detection capability. Mass spectrometry in an analytical technique widely involving the production and subsequent separation and identification of charged species according to their mass to charge ratio. The analytical approach is simple and rapid, yet reliable, achieving over the concentration range of 30 to 1000 ng/mL, sensitivity (limits of quantification = 15 and 10 ng/mL for Ketamine e NorKetamine), accuracy (90-104%), and precision (RSD < 8.1%) for all analytes [18,19]. The technique of GC / MS is a selective, sensitive and reliable, and is therefore considered a "gold standard" for determining the illicit drugs, medicines and psychoactive substances.

RESULTS

The single-phase test highlighted ten positive samples (3%) out of 294 patients involved in car accidents. The automated screening method confirmed only six out of ten samples resulted positive by single-phase test. All the patients were divided by age bands; we found positive results to ketamine in subjects between 18 and 47 years old. The 70% of the positivity belongs to the age band between 20 and 30 years old. For this reason, the 88 samples belonging to the age band 20-30 resulted negative by single-phase test, have been analysed with the automated instrument to perform a comparison between screening methods, highlighting any false negatives. The automated instrument found further four positivity [figure 1] out of 88 samples. The confirmation analysis were carried out by a gas chromatography-mass spectrometry instrument (GC / MS), with which we have analysed 14 samples resulted positive from both screening analysis. The presence of ketamine was confirmed by GC/MS only in seven samples out of fourteen [figure 2]. The GC/MS confirmed the 50% of positivity checked by single-phase test, five true positives out of 10. The GC/MS confirmed the 70% of positivity checked by automated instrument, seven true positives out of 10. Three of these were positive only to ketamine. All the positive samples confirmed were arrived at casualty with a Red Emergency Code. All the consumers were males.

The graphics on the right show the number of positive samples checked by the automated instrument: the upper graphic shows the samples found by the single-phase test and then confirmed by the automated instrument, while the graphic below shows other four positive samples checked in the age band between 20 and 30 years old (88 samples). The presence of ketamine is confirmed by GC / MS only in seven samples out of fourteen. Three of these were positive only to ketamine.

DISCUSSION

The reliability of the screening methods has been evaluated by analysing the positive results by GC/MS, which is the Gold Standard for the execution of toxicological analysis of medico-legal significance, also because it is a quantitative method. The reliability of the single-phase test results to be of 50% (five true positives, five false positives). Instead, the reliability of the automated method results to be of 70% (seven true positives, three false positives, four true negatives). The single-phase test (cut-off 1000 ng/ml) should have a high positive predictive value, so excluding false positives. The automated method (cut-off 330 ng/ml) should have a high negative predictive value, so discerning true negatives. Actually, the automated method does not confirm all the positive samples found by single-phase test, therefore the presence of false positives makes the single-phase test less reliable. Comparing the two screening methods, we obtain that higher sensitivity of the automated method is confirmed by the detection of four positive samples in a population group (88 samples) resulted negative by single-phase test. Moreover, the higher sensitivity is confirmed also by the presence of four samples true negatives confirmed by GC/MS. Those samples resulted positive by automated method but negative by GC/MS are quantitatively close to the cut-off value of the automated instrument (330 ng/ml); this probably justifies the false positives. We can conclude that the single-phase method, compared

with the automated instrument, overstate the positivity and exclude a part of positive samples (resulted false negatives) because it is under the cut-off value. Consequently, the single-phase method can only be used at an early stage of the toxicological test, so more analysis are necessary. The automated instrument has a higher reliability, because of the standardisation of methodology and analytical procedures. The cut-off value could be adjusted to increase the reliability. In the field of analysis requested by the New Italian Highway Code to patients involved in car accidents,

FIGURE 1. Comparison among methods.

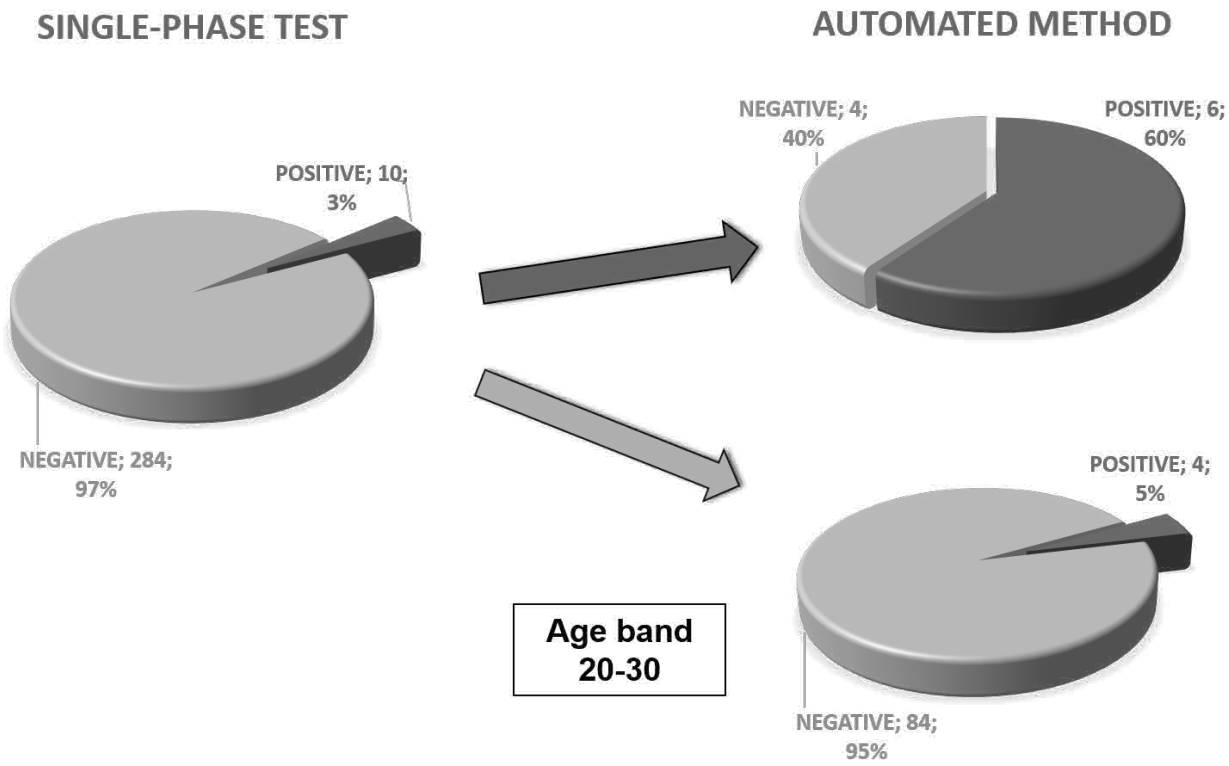
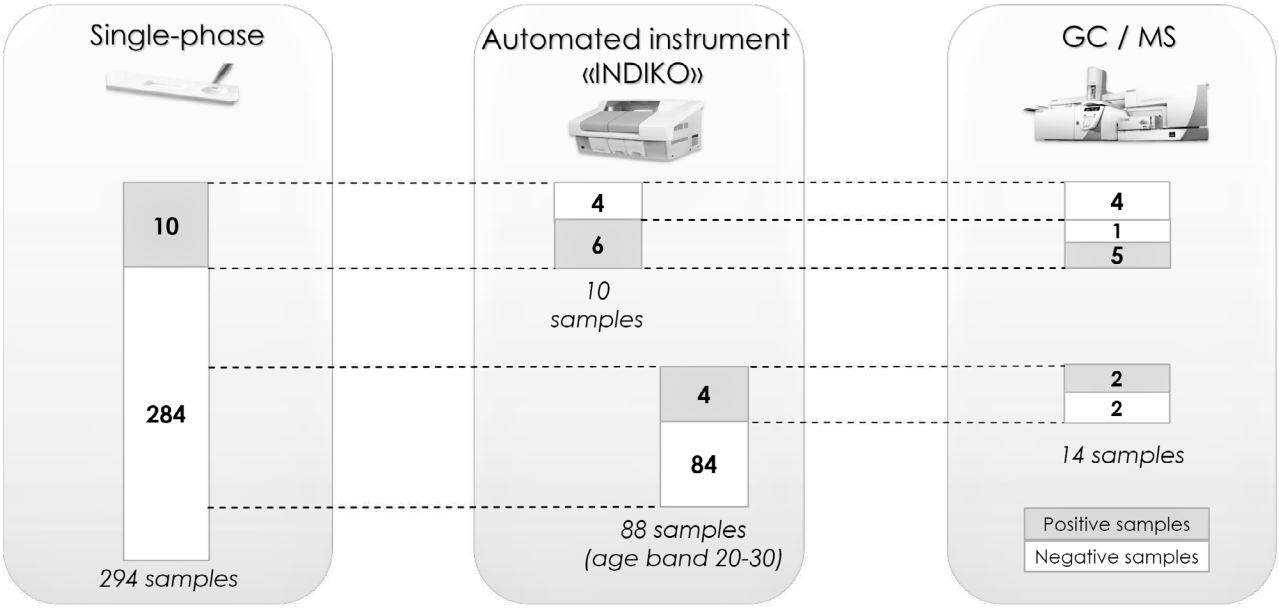


FIGURE 2. Comparison among single-phase test, automated screening method and confirmation test



a positive toxicological result provides a penalty and administrative sanction. Consequently, it is preferable to use a screening method more specific that can identify all the true negative samples and a limited percentage of false positives, which will be analysed by a confirmation test, that is the only one test with a medico-legal significance.

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