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Definitive Drug and Metabolite Screening in Urine by UPLC–MS-MS Using a Novel Calibration Technique

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Abstract

Drug screening is an essential analytical tool for detection of therapeutic, illicit and emerging drug use. Presumptive immunoassay screening is widely used, while initial definitive testing by chromatography-coupled mass spectrometry is hampered due to complex pre-analysis steps, long chromatography time and matrix effects. The aim of this study is to develop and validate a definitive test for rapid and threshold accurate screening of 33 drugs or metabolites (analytes) in urine. Sample preparation in a 96-well plate format involves rapid glucuronidase hydrolysis followed by dilution, filtration and ultra-performance liquid chromatography–MS-MS analysis. Chromatographic separation, on an ACQUITY UPLC® BEH phenyl column is optimized for a 3-min MS-MS ion acquisition. Matrix effect was normalized by an innovative technique called threshold accurate calibration employing an additional analysis with an analyte spike as an internal standard undergoing the same matrix effect as an analyte in a drug-positive donor specimen. Accuracy and precision, at above and below threshold concentrations, were determined by replicate analysis of control urine pools containing 50, 75, 125 and 150% of threshold concentrations. Accuracy and selectivity were further demonstrated by concordant findings in proficiency and confirmatory testing. The study shows the applicability of definitive testing as an alternative to immunoassay screening and demonstrates a new approach to normalization of matrix effect.

Introduction

Definitive drug testing is described by the American Medical Association as chromatography-coupled mass spectrometry methods of testing, either qualitative or quantitative, which provide specific identification of individual drugs or their metabolites (1). While definitive methods are used widely in confirmatory testing for forensic and sometimes for clinical toxicology casework, initial drug screening by definitive methods is limited in routine use to only a few subspecialties of toxicology practice. In postmortem toxicology, definitive screening methods have been used for many years as a standard of practice for identification of drug-related contributions to cause and manner of death. Traditional use of gas chromatography–mass spectrometry (GC–MS) for postmortem screening has progressed in recent years to the application of liquid chromatography–mass spectrometry (LC–MS) technology employing either nominal mass analysis in single (MS) or tandem (MS-MS) mode, or high-resolution mass spectrometry (2–9). Other areas of routine drug screening by definitive methods include therapeutic drug monitoring for pain management where clinical

compliance and abuse is monitored (10–13) as well as anti-doping evaluation where regulatory compliance of athletes is assessed (14–16). Drug screening in other areas of toxicology practice, including employment monitoring, court ordered surveillance, chemical dependency treatment and emergency toxicology has continued to rely heavily upon immunoassay methods for presumptive identification of a drug or drug class (17–19).

Experience has shown that multi-drug screening by definitive methods allows selective detection of illicit as well as therapeutic agents across many drug classes (3–9, 14, 20–30), while immunoassay methods provide only presumptive identification of a drug or more often a drug class (17–19). When used, frontline definitive testing has resulted in a reduced rate of false negatives compared with immunoassay screening (31–35). The reduced incidence of false negatives in definitive test screening is due in part to cross-reactivity and sensitivity issues. Definitive testing generally utilizes lower positive thresholds compared to immunoassays, which continues to employ thresholds established by governmental workplace drug testing. Pesce and coworkers documented a reduction of false negatives up to 69% in a large cohort of pain management patients, using definitive testing (31, 32). The highest incidence of false negative immunoassay testing was determined for hydromorphone, benzoylecgonine and alprazolam.

Dixon *et al.* reported substantial false negative rates for urine samples screened by an enzyme multiplied immunoassay technique (EMIT) benzodiazepine assay (33) owing to the relatively low cross-reactivity of the glucuronide metabolites. In an attempt to improve accuracy of the technique, they investigated incorporation of an additional glucuronide hydrolysis step to liberate parent drugs, but still reported false negatives of >35% in comparison with a LC–MS-MS method. They concluded that for compliance monitoring of patients receiving benzodiazepine therapy, immunoassay results should be used with caution and that chromatography-coupled mass spectrometry techniques are recommended. A more recent study by Passik *et al.* reported a similar false negative detection rate for benzodiazepines using immunoassay point of collection testing (34). The same study also reported significant false negative rates for other drug classes when data were compared with LC–MS-MS analysis, i.e. 43% false negative for amphetamines, 40% for cocaine, 29% for opioids other than methadone, 28% for methadone and 20% for marijuana. Melanson and coworkers used data from the College of American Pathologists (CAP) Proficiency Testing Surveys to assess and summarize some of the limitations of urine immunoassays for drugs of abuse testing (35). In addition to false negatives (particularly noted for MDMA and oxycodone), false positives were also observed. Finally, the cross-reactivity and ability of immunoassay to identify newer emerging psychoactive substances remains largely unclear (36–38).

While the potential for increased selectivity and sensitivity in definitive test screening is known, continuing use of immunoassay screening is due largely to the speed and automation of homogeneous immunoassay screening methods. Replacement of immunoassay by definitive screening has been technically hampered by labor-intensive extraction methods for sample preparation and by lengthy chromatographic run time. The need for sample derivatization by GC–MS has been overcome by the move to LC–MS based methods but sample preparation and chromatographic run time remain as challenges for the routine use of LC–MS technology in toxicology screening. While time and labor-intensive solid-phase extraction techniques for sample clean-up continue to prevail in LC–MS testing methods (20–23, 39), an increasing number of rapid dilute-and-inject methods have been developed and validated (14, 25, 26, 28, 30, 40, 41) in an effort to enhance the speed and convenience of definitive screening. In addition, the application of ultra-performance liquid chromatography (UPLC) with either reduced MS dwell time in nominal mass detection or rapid full spectrum acquisition in high-resolution MS analysis has allowed chromatographic separation in 5 min or less in a number of published methods for definitive urine drug screening (14, 28, 30, 39). Some laboratories have also adapted 96-well plate formats for sample preparation and analysis, allowing for automation and further applicability of definitive screening.

As convenience and speed of LC–MS based screening improves, matrix effects on ion entry into the MS source remain a challenge in the conversion to definitive screening in routine practice. It is known that electrospray ionization, which is the most commonly used method of ion preparation for LC–MS based analysis of drugs, is susceptible to matrix effects (42, 43). While the term “matrix effect” implies interference from endogenous component of urine, interference may also be produced by exogenous compounds such as other excreted medications or their metabolites, or by co-eluting compounds added as assay reagents. Proposed mechanisms of enhanced or reduced ionization by chromatographically co-eluting compounds include competition for either ion charges or droplet surface, viscosity effects on droplet formation and ion pairing with the analyte (44). Independent of the mechanism, it is generally agreed that matrix effect may be reduced by sample clean-up and chromatographic resolution (45, 46). In urine drug screening by definitive methods, however, the move toward rapid sample preparations and faster chromatography increases the potential for co-eluting interferents affecting analyte ion entry at the MS source. Inter-subject samples may have variable presence and concentration of co-eluting compounds that cause matrix effect. The threshold accuracy of screening by LC–MS(MS) will therefore be limited without either the elimination of interferents via sample preparation and chromatographic optimization or the normalization of the matrix effect by internal standardization.

Normalizing the matrix effect by inclusion of an internal standard is a routine practice in quantitative confirmation testing by LC–MS technology and stable-isotope-labeled internal standards (SIL-IS), when available, which are widely used for this purpose. While a few studies have shown that analyte-specific SIL-IS retention time may differ slightly from the analyte (45, 46) and that the use of non-analyte specific SIL-IS may not adequately compensate for matrix affect (42), analyte-specific SIL-IS is still regarded as the best approach to matrix normalization in quantitative testing by definitive methods. It is important to also note that qualitative screening by definitive methods as an alternative to immunoassay screening also requires matrix effect normalization in order to achieve threshold accurate drug detection for each urine sample tested.

We report the development of an UPLC–MS-MS method for screening of 33 drugs or metabolites (analytes) in urine using a two-sample preparation followed by rapid chromatographic analysis. The analytes panel includes opiates, opioids, benzodiazepines, amphetamines, amphetamine derivatives, cathinones, cocaine metabolite and hallucinogens. A rapid glucuronidase hydrolysis followed by dilution, filtration and UPLC–MS-MS analysis of dual samples is accomplished using a 96-well plate format. An innovative threshold accurate calibration (TAC) technique is used to normalize matrix effect and to attain threshold accuracy without the use of SIL-IS. Validation studies include precision and accuracy at above and below threshold concentration along with studies of matrix effect, detection limit, hydrolysis efficiency and correlation with other definitive methods.

Materials and methods

Technique principle

A novel technique (TAC) is used to achieve matrix effect normalization and threshold accurate detection of the 33 analytes. Figure 1 is a diagrammatic representation of the technique. TAC technique utilizes a procedure where analytes of interest are spiked into the sample to achieve a 100% of cut-off or threshold concentration as added standards. The TAC technique employs an analyte spike as an internal standard that undergoes the same matrix effect as an analyte in a drug-positive donor specimen. Each calibrator, control and donor-subject sample is analyzed twice, once without the added standards (neat sample) and once with the added standards (spiked sample). Following addition of recovery and hydrolysis reagent to both samples and, subsequent to incubation, the hydrolyzed neat and spiked sets are diluted, filtered and analyzed by UPLC–MS-MS.

A TAC ratio is calculated for each analyte by the following equation: (ion area of the neat sample analysis)/(ion area of the spiked sample analysis − ion area of the neat sample analysis). The TAC ratio is calibrated by analysis of a urine specimen containing threshold concentration of each analyte. Positive analyte detection in quality control (QC) and test samples is determined by a TAC ratio that exceeds the calibrator TAC ratio. Figure 1 depicts the analysis of calibrator urine along with threshold negative and positive test samples. Multiple analytes may be tested by the use of multi-analyte calibrator and spike reagents as employed in the assay being reported.

Chemicals, supplies and reference material

ACS grade methanol, HPLC grade acetonitrile and isopropanol were purchased from Fisher Scientific (Fairlawn, NJ). Reagent grade ammonium formate (97%) and formic acid (>95%) were obtained from Sigma-Aldrich (St Louis, MO). Morphine-3-beta-D-glucuronide (M3G) was purchased from Cerilliant (Round Rock, TX), methapyrilene HCl from Supelco Analytical (Bellefonte, PA) and the analytes listed in Table I were obtained from either Cerilliant and/or Grace (Deerfield, IL) for preparation of separate calibrators and controls. Purified beta glucuronidase (IMCSzyme, activity ≥50,000 U/mL) and beta glucuronidase buffer (IMCSzyme) was obtained from and certified by Integrated Micro-Chromatography Systems (Columbia, SC).

Sirocco™ protein precipitation plates, 2 mL square collection plates and an extraction plate manifold were obtained from Waters Corporation (Milford, MA). Analyte-negative urine used for preparation of calibrator and QC samples was obtained from laboratory volunteers and the absence of detectable analytes was verified by LC–MS-MS analysis. Deionized water was produced by combined ultra-filtration (AquaPure, Schenectady, NY) and reverse osmosis (Alpha Water Systems, Montague, NJ) treatment.

Reagents and controls

Two sources of stock multi-analyte reference solutions were prepared in methanol from separate sources of reference material and stored at −10°C. Stock concentrations were prepared at 10, 2.5 and 1.0 µg/mL concentrations, respectively, for analytes with thresholds of 100, 25 and 10 ng/mL as specified in Table I. Multi-analyte calibrator urine was prepared on the day of analysis by dilution of stock reference material with analyte-negative urine at the analyte-specific threshold concentrations listed in Table I. Working QC samples for testing were prepared on the day of analysis by dilution of the stock multi-analyte reference solution with analyte-negative urine to achieve concentrations of 75, 125 and 400% of threshold. Additional QC samples with 50 and 150% of threshold concentration for multi-analytes were prepared and analyzed as part of the validation plan.

A stock solution for hydrolysis control containing 10 µg/mL of M3G was prepared in methanol and stored at −10°C. Hydrolysis control urine for testing was prepared on the day of analysis by dilution of the stock hydrolysis control in analyte-negative urine at a free-analyte equivalent equal to 400% of threshold concentration. Multi-analyte spike reagent was prepared at 400% of analyte threshold concentrations by dilution with analyte-negative urine. As an injection recovery standard, a stock solution of methapyrilene was prepared in methanol at 10 µg/mL and stored at −10°C. On the day of analysis, a working recovery reagent containing 200 ng/mL of methapyrilene was prepared by dilution of stock methapyrilene in

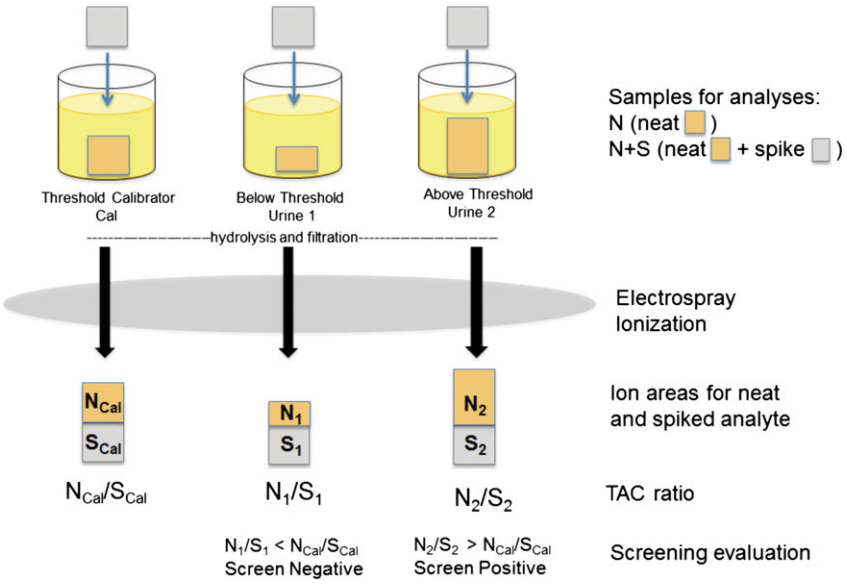

Figure 1. Schematic representation of TAC technique for drug screening in human urine.

Table I. Summary of analyte and recovery standard retention time, transition ions, dwell times (msec), cone voltage (V) and collision energies (eV) for UPLC–MS-MS analysis[a]

	Retention time (min)	Cone voltage/dwell time (msec)	Target transition ions (collision energy)	Qualifier transition ions (collision energy)	Threshold (ng/mL)
6-Acetylmorphine	1.4	45/5	328.1 > 58.2 (35)	328.1 > 165.1 (35)	10
α-hydroxyalprazalam	2.4	51/5	325.2 > 216.2 (41)	325.2 > 297.2 (23)	100
Alprazolam	2.5	50/5	309.1 > 281.2 (27)	309.1 > 205.2 (42)	100
Amphetamine	1.3	20/5	136.1 > 91.2 (15)	136.1 > 119.1 (8)	100
Benzoylecgonine	1.8	39/5	290.2 > 168.1 (19)	290.2 > 105.1 (30)	100
Buprenorphine	2.1	60/5	468.4 > 55.1 (70)	468.4 > 84.2 (70)	10
Clonazepam	2.4	50/5	316.1 > 270.2 (25)	316.1 > 214.3 (35)	100
Codeine	1.4	50/5	300.1 > 165.3 (35)	300.1 > 153.2 (35)	100
Diazepam	2.6	55/100	285.1 > 154.2 (29)	285.1 > 193.2 (29)	100
EDDP	2.1	47/100	278.3 > 186.2 (33)	278.3 > 249.2 (23)	100
Flurazepam	2.1	45/25	388.2 > 315.2 (21)	388.2 > 100.2 (29)	100
Hydrocodone	1.5	60/5	300.4 > 199.2 (35)	300.4 > 128.1 (55)	100
Hydromorphone	1.1	60/5	286.4 > 185.3 (30)	286.4 > 157.2 (40)	100
Lorazepam	2.4	41/5	321.1 > 275.2 (21)	321.1 > 303.2 (21)	100
MDA	1.4	20/5	180.4 < 163.1 (15)	180.4 > 105.1 (20)	100
MDEA	1.5	30/50	208.3 > 163.2 (13)	208.3 > 105.2 (25)	100
MDMA	1.4	27/5	194.2 > 163.2 (13)	194.2 > 105.2 (25)	100
MDPV	1.8	35/5	276.2 > 175.2 (20)	276.2 > 135.2 (30)	100
Mephedrone	1.5	25/5	178.2 > 160.2 (15)	178.2 > 145.2 (20)	100
Methadone	2.3	33/5	310.3 > 105.1 (28)	310.3 > 57.2 (25)	100
Methamphetamine	1.4	27/5	150.2 > 91.2 (17)	150.2 > 119.2 (11)	100
Methapyrilene	1.9	25/5	262.3 > 97.1 (35)	262.3 > 217.3 (20)	100
Methylone	1.3	30/5	208.2 > 160.2 (20)	208.2 > 132.3 (30)	100
Midazolam	2.2	55/25	326.2 > 291.3 (26)	326.2 > 244.3 (26)	100
Morphine	0.9	50/100	286.4 > 44.1 (31)	286.4 > 165.3 (40)	100
Norbuprenorphine	2	70/25	414.2 > 57.1 (43)	414.2 > 101.1 (40)	10
Nordiazepam	2.5	55/5	271.1 > 140.1 (29)	271.1 > 165.1 (27)	100
Oxazepam	2.5	39/5	287.1 > 241.2 (23)	287.1 > 269.1 (13)	100
Oxycodone	1.4	40/5	316.3 > 241.3 (30)	316.3 > 212.3 (40)	100
Oxymorphone	1	43/100	302.2 > 227.2 (31)	302.2 > 198.2 (45)	100
Phencyclidine	2	19/5	244.3 > 86.2 (11)	244.3 > 91.2 (27)	25
Temazepam	2.5	30/5	301.1 > 255.2 (22)	300.1 > 177.2 (40)	100
Tramadol	1.7	31/5	264.3 > 58.1 (21)		100
Triazolam	2.5	56/5	343.2 > 308.2 (26)	343.2 > 239.2 (44)	100

[a]Also listed are the analyte threshold concentrations (ng/mL) used in calibrating the screening analysis.

analyte-negative urine. Buffered hydrolysis reagent containing 60% beta glucuronidase buffer and 40% purified beta glucuronidase was also prepared on the day of analysis.

Analytical method

Sample preparation

Two samples (200 µL each) of calibrator, QC, hydrolysis control and test urine specimens were added to paired analysis wells in a 96-well plate for neat and spike analysis. Multi-analyte spike reagent (50 µL) was added to spike wells and negative urine (50 µL) was added to neat wells, followed by additions of working recovery reagent (50 µL) and buffered hydrolysis reagent (50 µL) to all wells. The wells were mixed and incubated at 55°C for 1 h in an oven. After cooling, 600 µL of starting mobile phase was added to all analysis wells and mixed. The samples were filtered in the extraction plate manifold by the application of negative pressure, and 5 µL of filtrate was analyzed by UPLC–MS-MS.

UPLC–MS-MS analysis

UPLC–MS-MS was performed using a Waters ACQUITY UPLC I-Class (FTN) system in combination with a Xevo® TQD tandem mass spectrometer (Waters Corporation, Milford, MA). Chromatographic separation was achieved using an ACQUITY BEH Phenyl column (1.7 µm, 2.1 × 50 mm, Waters) maintained at 45°C. The mobile phase solvent A was 2 mM ammonium formate in water containing 0.1% formic acid and solvent B was 2 mM ammonium formate in methanol containing 0.1% formic acid. The flow rate was 0.6 mL/min and a gradient elution was applied as follows: 2% B (0–0.5 min), 2–70% B (0.5–2.2 min), 70–90% B (2.2–2.7 min) and 90–2% B (2.7–3.0 min) with a 0.3-min hold. The injection wash solvent contained isopropanol:acetonitrile:water (1:1:1).

The mass spectrometer was operated in electrospray positive ionization mode using the following MS-MS conditions: capillary voltage (0.55 kV), source temperature (150°C), desolvation gas (nitrogen delivered at a flow rate of 1,000 L/h and at a temperature of 550°C), collision gas (argon maintained at a pressure of 0.5 Pa) and RF 2.5 V. Multiple reaction monitoring (MRM) was performed for the 33 drugs and metabolites using two transitions per analyte (with the exception of tramadol for which only one transition was monitored); cone voltage and collision energy were optimized to give the maximum response for each transition (Table I). Parameters were arranged into 33 individual acquisition windows with individual dwell times (also shown in Table I) optimized to provide

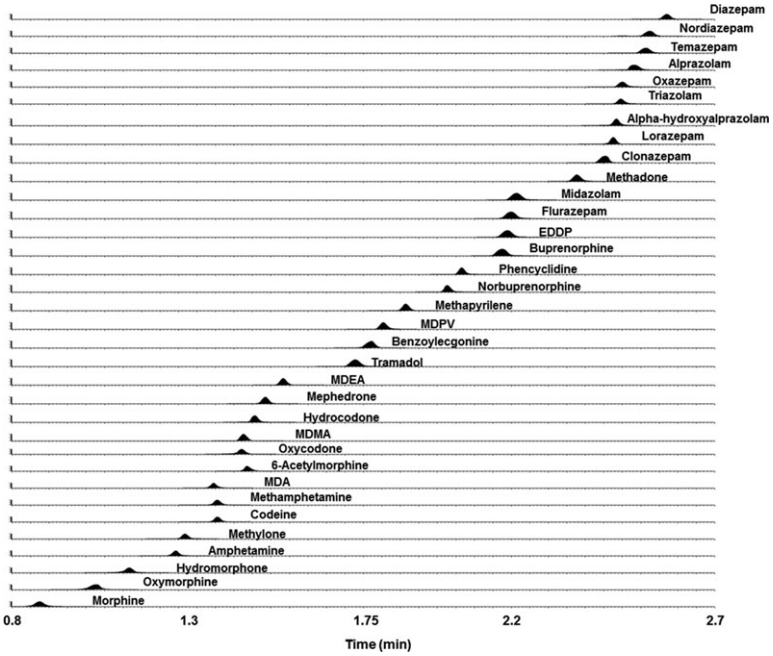

Figure 2. Total ion chromatogram of target transition ion for 33 drug and metabolite analytes in 3-min acquisition program for a calibrator urine filtrate.

accurate profiling of the chromatographic peaks (typically >15 points/peak). Target transition ion ratios (i.e. qualifier response/quantifier response) were determined from the average obtained with the calibrator and the QC urine samples at 75, 125 and 400% of threshold concentration.

Data processing

Data were processed initially using TargetLynx™ (Waters) which integrated peak-area responses for each MRM transition and verified acceptability of results with respect to several QC criteria including a retention time within ±0.1 min of the retention time established by reference analyte analysis and transition ion ratios within 20% of target for ratio >0.50, within 25% of target for ratio <0.5–0.20, 30% of target for ratio <0.20–0.10 and 50% of target for ratio <0.10. For each injection, verification of a successful injection process was achieved by examination of the target ion peak-area responses of the recovery standard, i.e. methapyriline.

Subsequent data handling was achieved by export of the TargetLynx summary report into Microsoft Excel, which used quantifier ion peak-area response to calculate and establish the "TAC" ratio (Figure 1). The TAC ratio is calculated for each analyte in each specimen as follows: TAC ratio = neat peak-area/(spiked peak-area − neat peak-area). The TAC ratio was calculated using a single urine calibrator containing all drugs at their respective threshold concentration. QC and test sample TAC ratios are compared to the corresponding analyte TAC ratio determined for the calibrator urine, and analytes with a TAC ratio exceeding the calibrator TAC ratio are reported as positive if retention time, injection recovery and ion ratio criteria are met.

Screening method validation plan

The validation plan was based upon both SWGTOX guidelines (47) and Laboratory Standards provided by New York State's Department of Health (48). Threshold accuracy and precision were determined by replicate analysis of QC samples containing 50, 75, 125 and 150% of threshold analyte concentration. Matrix effect was determined by analysis of filtered negative urine supplemented with 50–200% threshold concentration of analyte (B) compared with analyte supplemented in starting mobile phase at same concentrations (A). The percent matrix effect was calculated using the following formulae: (B/A − 1) × 100%. Analyte-negative urine from multiple-donor collections ($n = 10$) was analyzed to evaluate the presence of any co-eluting interferents. Hydrolysis efficiency was assessed on each analytical run by inclusion of a glucuronidated analyte (M3G) at concentration above threshold. Qualitative accuracy of the positive results by the definitive screening method was assessed by co-analysis of New York State's Department of Health proficiency testing specimens and de-identified positive case specimens, using validated confirmation testing by quantitative LC–MS-MS methods. Specimen and data handling for de-identified positive and negative case specimens were based upon an Albany Medical Center IRB protocol. For evaluating the effect of urine concentration on matrix effect, urine creatinine was measured on an MGC240 analyzer (Microgenics Corp. Fremont, CA) using a validated alkaline picrate method. Immunoassays for opiates, oxycodone, buprenorphine, cocaine metabolite, amphetamine, MDMA, benzodiazepine and phencyclidine were performed by a validated Microgenics Corp. assay on an MGC240 analyzer. Confirmation testing was performed by validated LC–MS-MS methods.

Results and discussion

Analytical column and gradient mobile phase conditions were selected for optimal retention of morphine and separation of analytes, especially isobaric agents, in a rapid chromatographic run time. Transition ion chromatograms for the 33 analytes and recovery standard with finalized gradient conditions are shown in Figure 2. With optimization of mobile phase, the phenyl column showed adequate separation of morphine from the void volume with baseline separation of the isobaric analytes morphine/hydromorphone and

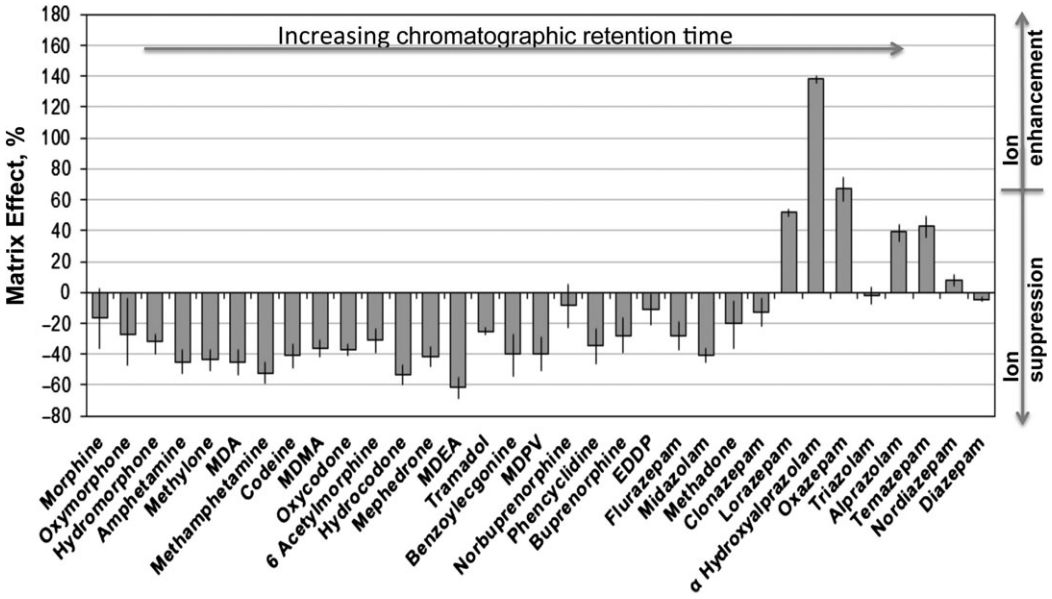

Figure 3. Matrix effect (mean and SD) determined by analysis of seven negative urine pools spiked with 50–200% threshold concentration of analyte compared with analyte-spiked mobile phase at the same concentration. Matrix effect = 100 × ((urine spiked ion area/mobile phase spiked ion area) −1).

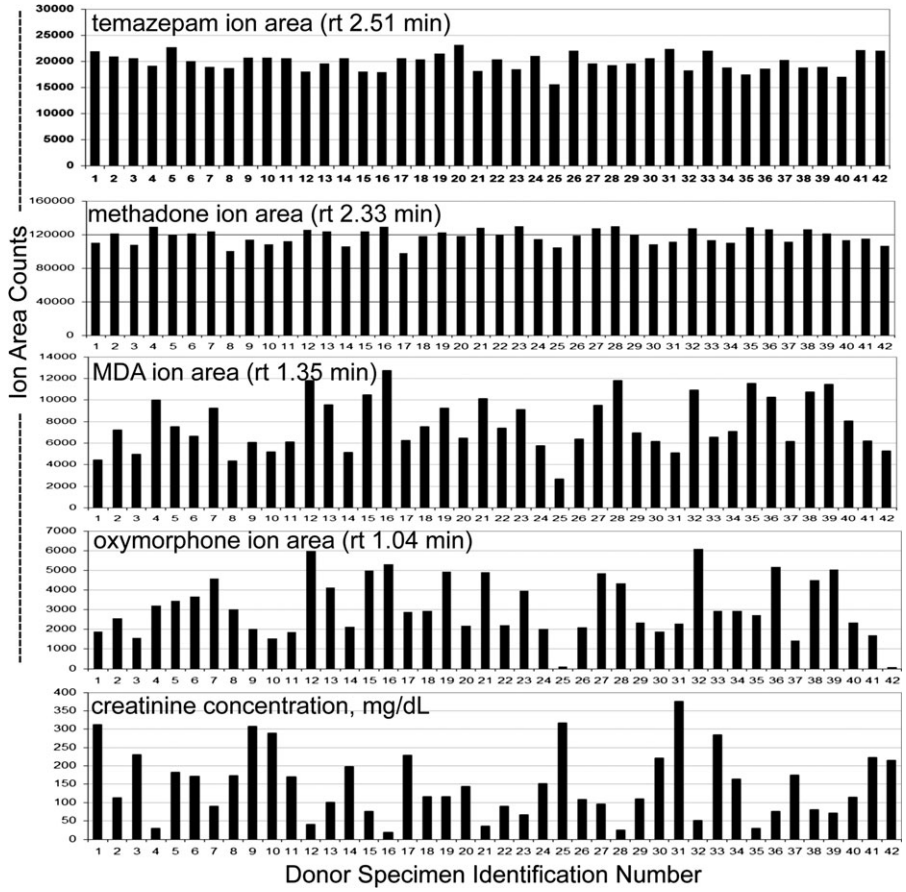

Figure 4. Variation in urine creatinine concentration and target ion area for oxymorphone, MDA, methadone and temazepam in 42 negative urine specimens supplemented with 100 ng/mL concentration of analytes.

codeine/hydrocodone. Other analytes that are either closely eluting or co-eluting have transition ion selectivity and analytical resolution. Except for isobaric analytes, no additional chromatographic peaks are observed in the analyte-acquisition windows and analysis of multiple analyte-negative urine specimens did not demonstrate the presence of interferents for any of the 33 analytes.

Matrix effect was evaluated in multiple-donor urine specimens in order to evaluate ion suppression or enhancement in the described dilute-and-filter method of drug screening. Matrix effects varied widely between analytes ranging from 61% suppression to 139% enhancement. In Figure 3 the mean and standard deviation of percent matrix effect is represented in relation to an increasing order of retention time. Results show a pattern of ion suppression for early- and mid-chromatography eluting analytes and ion enhancement for most late-chomatography eluting analytes. This finding is consistent with observations of others (27) and with the mechanisms proposed as the primary cause of matrix effects, i.e. co-elution of other molecules (organic or inorganic) resulting in competition at the ion source and consequent reduced or enhanced response of the analyte ions (44, 49, 50). It would be predictable, therefore, that the concentration of co-eluting compound and the resultant degree of matrix effect for each analyte may vary between urine specimens.

The variability of matrix effect between urine specimens was evaluated further by supplementing 42 analyte negative urine donor specimens with an equivalent concentration of analyte with co-analysis of creatinine concentration as an index of overall urine concentration. Figure 4 shows the resulting transition ion area for representative early and late eluting analytes. For the early eluting oxymorphone and MDA, ion area varied with coefficients of variation of 46 and 36%, respectively, and the pattern of data suggested a correlation of low analyte ion area and high creatinine concentration. For the relatively late eluting methadone and temazepam, the inter-specimen variability of ion area was <9% and a pattern of relationship with creatinine concentration was not evident. Regression line analysis was performed using analyte ion area and creatinine concentration for a series of analytes with chromatographic retention time <1.5 min (Figure 5) and >1.5 min (Figure 6) in the 42 donor urine analyses. A pattern of decreasing ion area with increasing creatinine concentration is observed consistently for the early eluting analytes and not for later eluting analytes. The data support the prediction of inter-specimen variability in matrix effect for each analyte, which may be chromatography time and urine concentration dependent. The underlying mechanism may be a concentration-dependent competition for ionization or ion entry at the source by co-eluting compounds and this may help to explain the value of dilution in drug screening by LC–MS technology. On a practical basis, it further emphasizes the need for normalization of matrix effects for accuracy in drug testing.

While matrix effect varied widely across the analytes and between urine specimens, normalization of the effect was achieved

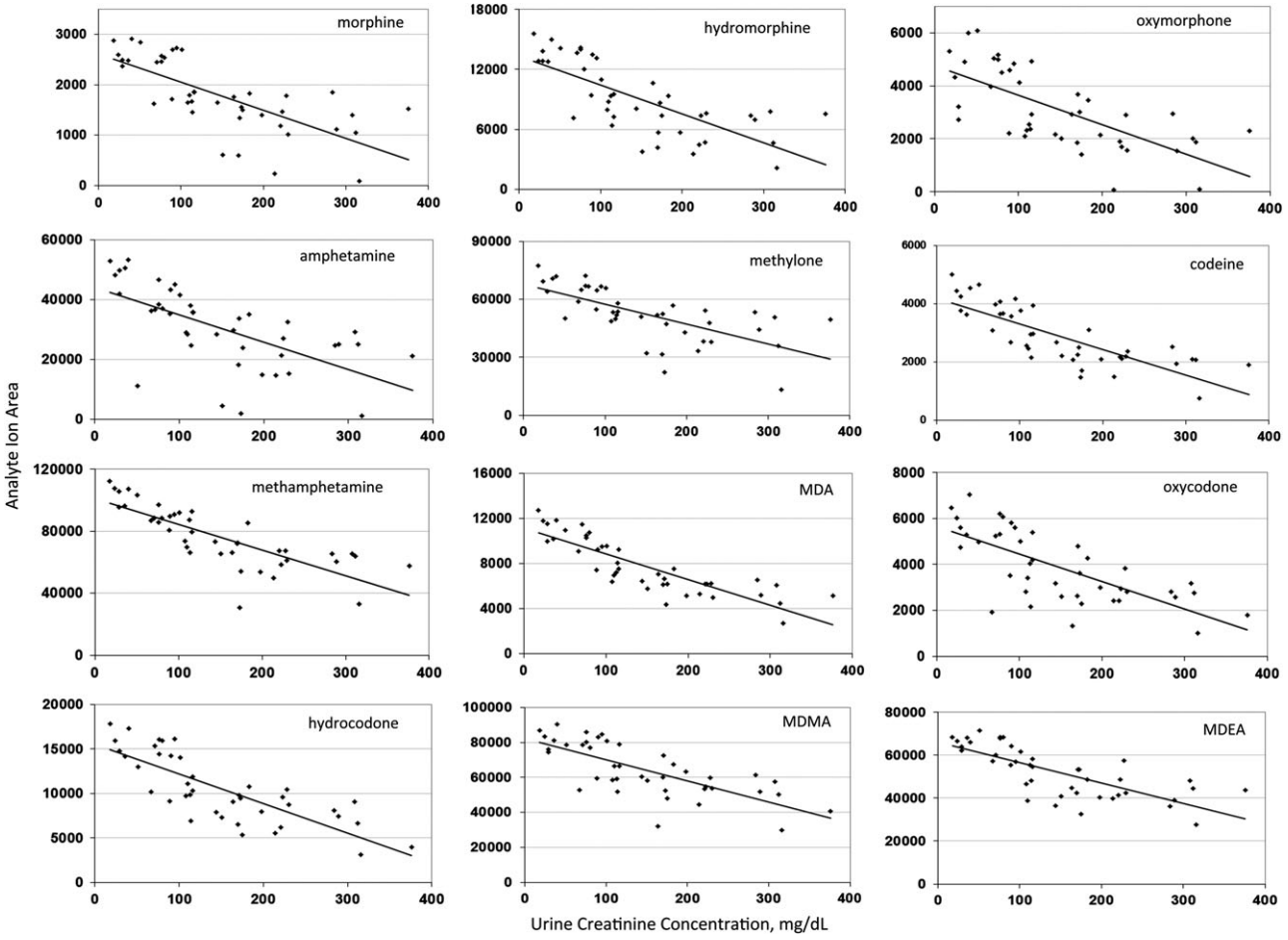

Figure 5. Effect of urine concentration on ion suppression of analytes with chromatographic retention time <1.5 min. Negative urine from 42 donors was spiked at threshold concentration of analytes and analyzed for drug, metabolite and creatinine concentrations.

by application of the TAC technique. Figure 7 shows the precision and limited variation in the matrix-normalized TAC ratio for calibrator urine prepared in multiple-donor urine matrices. The accuracy of screening by the TAC technique is demonstrated in Figure 8 for replicate QC analyses at threshold surrounded targets of 50, 75, 125 and 150% of threshold concentration. Accuracy averaged 99.0% with a range of 90.5–117% across all analytes. The corresponding QC precision averaged 8.9% with a range of 2.3–17.8% for all analyte data (Figure 9). The incubation time for glucuronidase hydrolysis was optimized by testing the hydrolysis control at incubation times of 15, 30, 60, 120 and 180 min. Maximum hydrolysis and recovery of free drug was achieved in 1 h. Inclusion of the hydrolysis control on each analytical run demonstrates the consistency of hydrolysis efficiency over multiple analytical runs.

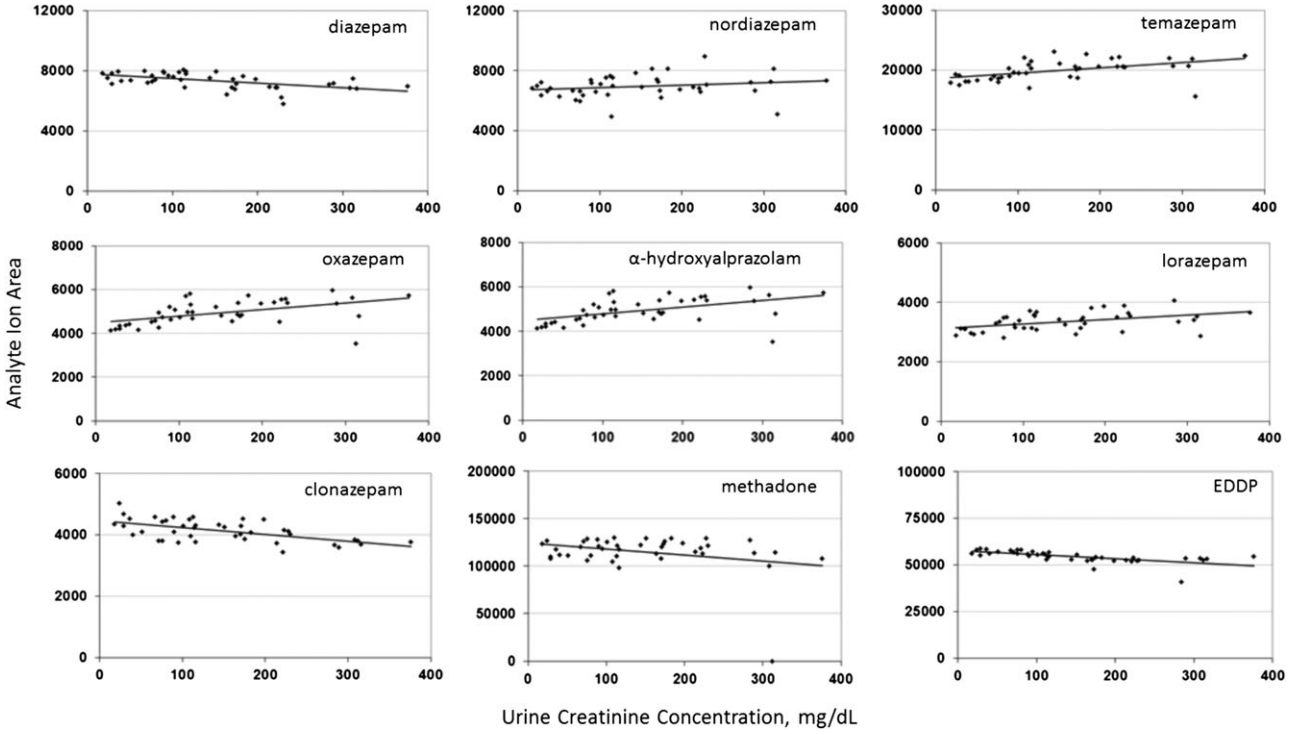

Figure 6. Effect of urine concentration on ion suppression of analytes with chromatographic retention time >1.5 min. Negative urine from 42 donors was spiked at threshold concentration of analytes and analyzed for drug, metabolite and creatinine concentrations.

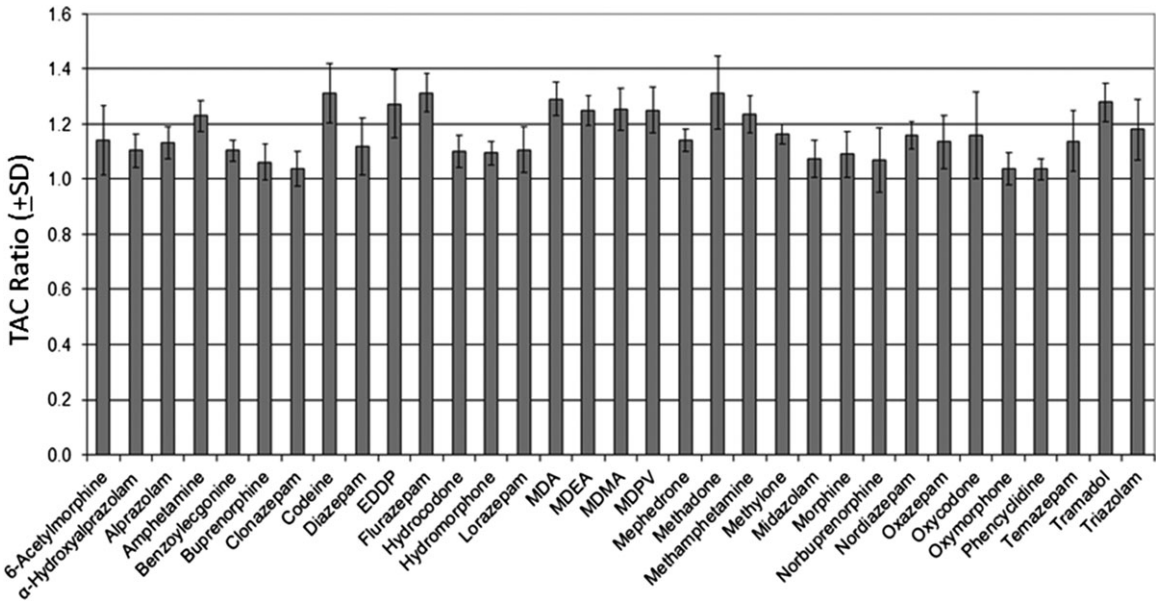

Figure 7. TAC ratio (mean ± SD) for analyzed of calibrators prepared in seven negative urine pools by supplementing with reference standard material at threshold concentration for 33 analytes.

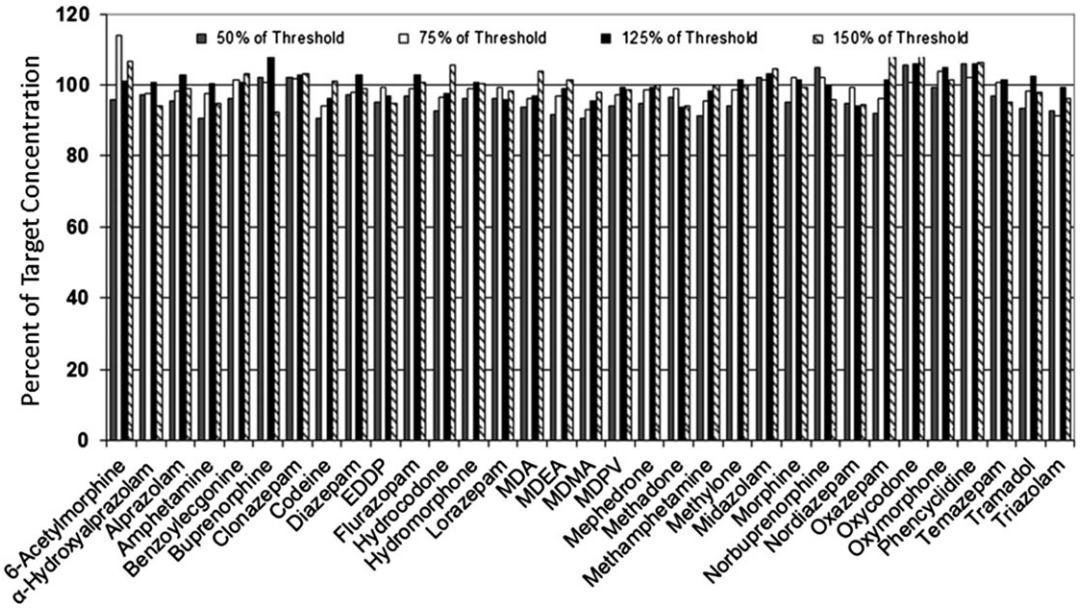

Figure 8. Percentage of target concentration within and between run analysis data for multi-sourced negative urines supplemented with 50–150% of threshold concentration (mean 99.0%, range 90.5–117%).

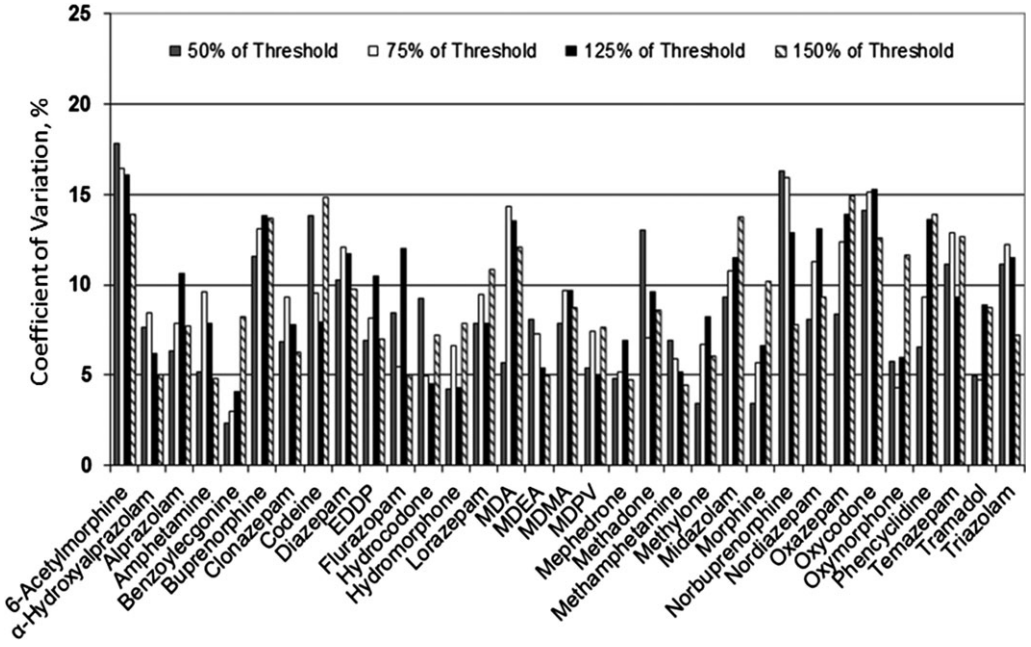

Figure 9. Coefficient of variation for within and between run analysis data for multi-sourced negative urines supplemented with 50–150% of threshold concentration (%CV mean 8.9%, range 2.3–17.8%).

Method correlation studies were performed using immunoassay screen-positive specimens resulting in 178 drug and metabolite finding by currently validated definitive confirmation methods. Specimens include de-identified specimens from clinical and forensic casework as well as proficiency testing urine specimens from New York State's Department of Health. Complete concordance was found with case and proficiency specimen findings by definitive screening and confirmation testing as shown in Figure 10. Evaluation of the incidence of false negatives by immunoassay was not possible due to the use of a selected cohort of screen-positive cases. Definitive screening did, however, reveal the specific drugs and metabolites versus drug classes detected by immunoassay and also detected additional drug use in casework samples including methadone, tramadol, carisoprodol and methylphenidate.

Conclusions

A UPLC–MS-MS method has been developed and validated for multi-analyte screening in urine. Application of definitive drug and metabolite screening by convenient sample preparation and rapid

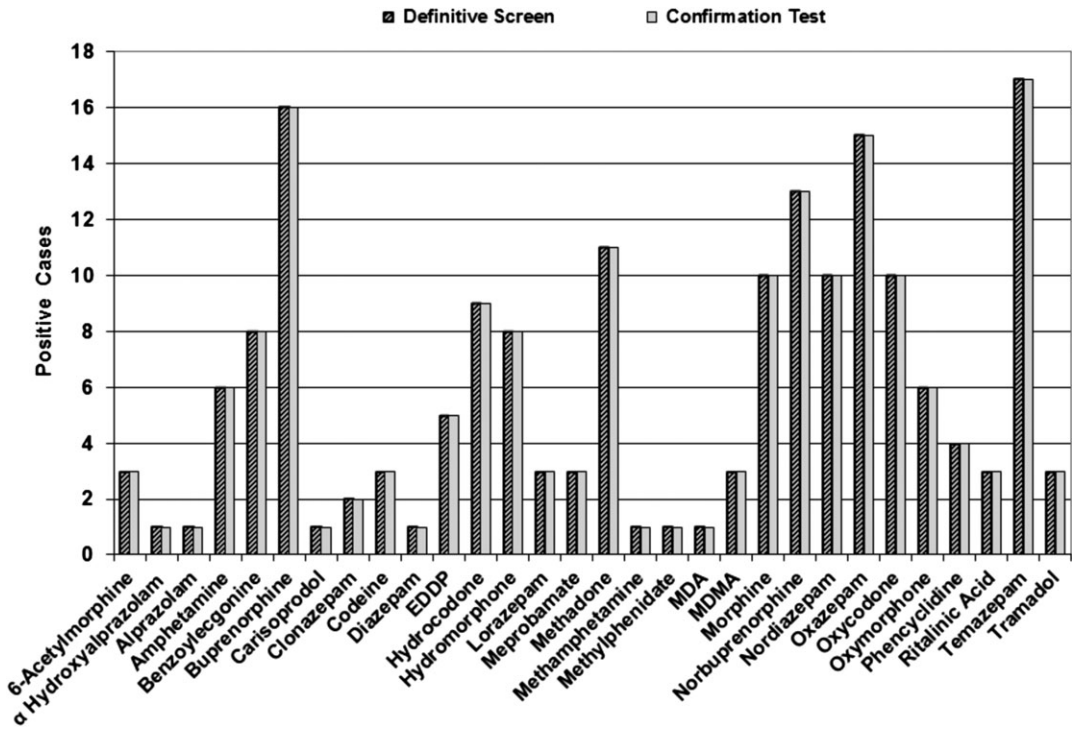

Figure 10. Comparison of positive analyte finding ($N = 178$) by definitive screening method in proficiency and case specimens compared with analyte detection by confirmatory LC–MS-MS testing.

chromatographic analysis has been demonstrated and provides an alternative to presumptive immunoassay screening. In addition, a novel calibration technique (TAC) has been developed for matrix effect normalization and threshold accurate screening as an alternative to SIL-IS. While the TAC technique does requires two injection, the total run time per case is >7 min and the method is adaptable to accurate, high volume screening. With the rapidly growing number of new psychoactive agents and designer drugs, new techniques for accurate and rapid definitive drug and metabolite screening without the need for SIL-IS may be applicable in the evolving practices of clinical and forensic toxicology.

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