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RESEARCH ARTICLE

Evaluation of Diagnostic Sensitivity and Specificity of a New Automatized Assay for Indirect Synthetic Urine Identification

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ABSTRACT

Synthetic urine mimicking normal urine is difficult detectable by standard specimen validity testing. In this study, the VDx True Urine LD Test and True Urine SD Test, analysing 'long duration' (LD) and 'short duration' (SD) markers, were evaluated for their ability to detect synthetic urine. Two synthetic urines from the US market and 1188 real-life urine specimens were analysed for LD markers, SD markers, uric acid and temperature. Forty-seven specimens (set 1) showed uric acid and/or temperature suspicious results and were analysed by LC–MS/MS for 10 endogenous biomolecules. Diagnostic sensitivities and specificities were calculated for LD and SD markers based on uric acid and LC–MS/MS results of set 1. The false-positive rate of the SD marker was further evaluated with uric acid results of the other 1141 specimens (set 2). Additionally, direct synthetic urine markers were analysed by LC-(HR)MS. Uric acid and LC–MS/MS could identify synthetic urine. In one US synthetic urine, low concentrations of uric acid were found. Specimens of the Austrian/German region were reliably classified congruently by both methods. The LD and the SD markers detected both synthetic urines. For set 1, the LD had 100% sensitivity and 88.9% specificity, and the SD had 84.2% sensitivity and 44.4% specificity for authentic/synthetic specimens. In set 2, the SD marker specificity depended on the upper limit of the acceptance criteria. In one US synthetic urine, carbendazim was identified. Overall, several tests should be applied to confirm authentic or synthetic urine. Especially, simultaneous analysis of indirect and direct synthetic urine markers should be considered.

1 | Introduction

Forensic and clinical toxicology plays a crucial role in detecting drug and alcohol use by analysing various biological matrices such as blood, urine, oral fluid and hair [1–4]. Urine is often preferred for drug abuse testing due to its ease of collection [1, 5]. However, individuals may manipulate urine samples to avoid detection, particularly in workplace or abstinence programs. Common strategies include diluting the sample, tampering with the sample to interfere with the analytical method or substituting the urine specimen with a different liquid, for example, synthetic urine (SU) [6–10]. SU mimics the physiological properties of real urine, such as creatinine (CREA) concentration, pH and specific gravity (SG) [9, 11–13]. Thus, SU may pass standard specimen validity testing (SVT) given by national and international guidelines [14–17].

A manipulation attempt by SU can be detected indirectly by analysing endogenous biomolecules (EB) or exogenous

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substances (e.g. caffeine) present in real urine but absent in SU [8, 12, 18]. Small EB (< 1 kDa) analysed for urine identification/authenticity testing comprise, for example, CREA, uric acid (UA), urobilin, amino acids (metabolites) and acylcarnitines [12, 13, 19, 20]. Different presumptive colorimetric assays including strip tests as well as automatized procedures [21–23] and confirmatory liquid chromatography coupled with (tandem) mass spectrometry (LC–MS/MS)-based techniques [12, 18, 19, 24] targeting small EB were developed and evaluated for routine application [9, 13, 21, 23]. Nevertheless, macromolecules, such as urinary tract proteins (UTP), may also function as target analytes for urine identification. Tamm–Horsfall protein (THP) is the target analyte of an immuno-chromatographic lateral flow strip test intended for identification of human urine in forensic case work [25–27]. However, THP was found to be unstable by other studies [20, 28]. Recently, a panel of different automatized assays was introduced including but not limited to UTP as target analytes for urine authenticity testing [29].

Besides these indirect approaches, SU may also be detected directly by analysing SU-specific components. Novel SU markers in several SU products have been identified by LC–MS/MS, especially glycol-based substances (e.g. polyethylene glycols [PEG], polypropylene glycols [PPG] and putatively hydroxylated PPG [PPG + 16]) [12, 13, 19]. Analysis of a biomarker panel including urinary endogenous/exogenous and SU-specific compounds was found to be the most reliable approach in identifying SU by previous studies [9, 13, 19].

The first aim of this study was to evaluate the diagnostic sensitivity and specificity of a novel commercially available automated analyser assay for SU/urine adulteration detection, which targets UTP referred to as 'long duration' (LD) and 'short duration' (SD) markers [29] in Austrian real-life urine specimens. The second aim of this study was the investigation of SU from the US market (US-SU) with LC-high resolution (HR) MS for the identification of new direct SU markers.

2 | Experimental

2.1 | Chemicals and Materials

The DRI Creatinine Detect Test was procured from Microgenics Corporation (Fremont, USA) and the urea and UA detection kits from Beckman Coulter (Brea, USA). The True Urine LD Test (targeting long duration UTP-IV) and the True Urine SD Test (targeting short duration UTP II and III), two components of the VDx GEN2-Validity Panel, were kindly provided by Validity Diagnostics (Branford, Florida, USA). The Surestep Urine Adulteration Test Strip was procured from Abbott (Cologne, Germany). Ammonium format was purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN), formic acid and water were purchased in LC–MS-grade from Fisher Chemical (Schwerte, Germany). Two SU products from the US market were provided by Validity Diagnostics: Upass Synthetic Urine (US-SU1) (Urine World) and Quick Fix Plus 3 oz Synthetic Urine (US-SU2) (Spectrum Labs, Las Vegas, USA). An analytical standard for carbendazim was obtained from Sigma-Aldrich (St. Louis, USA).

2.2 | Methods

2.2.1 | Urine Specimens

Urine specimens ($n = 1188$), submitted to the authors' laboratory for drug testing, were measured for temperature immediately after specimen collection (if available) with an infrared thermometer (Fluke 62 Max). CREA, urea, UA, LD, and SD markers were analysed in both US-SUs and within all 1188 urine specimens, usually within 48 h after urine collection. Depending on temperature and UA results, urine specimens were assigned either to the suspicious specimen set 1 ($n = 47$) or to the unsuspicious specimen set 2 ($n = 1141$). Specimens were assigned to specimen set 1 if the temperature was < 33.5°C or > 37.9°C and/or if UA was not detectable (UA < 10 µmol/L, limit of detection [LOD] according to user manual). Set 1 specimens were frozen before they underwent additional testing procedures, including adulteration test strip and LC–MS analysis. Test persons consented to (follow-up) analysis.

2.2.2 | Test Procedure 1 (TP1)—Standard Specimen Validity Testing

TP1 included determination of CREA, urea, pH, SG and adulterants. CREA (Jaffe reaction, linearity of the test: 7.8–4200 mg/L according to user manual) and urea (enzymatic detection, linearity of the test: 10–750 mmol/L according to user manual) were analysed on an AU480 auto-analyser (Beckman Coulter, Krefeld, Germany). Physiological CREA values in urine are 260–2170 mg/L for females and 390–2590 mg/L for males [15]. In this study, CREA concentrations of ≥ 200 mg/L were evaluated as passed as given by Brenner–Hartmann and SAMHSA [14, 15, 17]. For urea, to the author's knowledge, there are no reference values for single time-point collected urine specimens available. In a previous study, urea concentrations in single collected authentic urine specimens with CREA concentrations ≥ 200 mg/mL were between 65 and 583 mmol/L [13]. Therefore, urea analysis was evaluated not passed when urea was not detectable (LOD = 5.71 mmol/L for urea according to user manual) and found low when < 65 mmol/L. Urine samples were added to the reagent pads of the Surestep Urine Adulteration Test Strips including CREA, nitrite, glutaraldehyde, pH, specific gravity (SG) and oxidants/pyridinium chlorochromate. Colour changes were compared to the colour legend given by the manufacturer after 1 min incubation (in the following referred to as 'test strips'). TP1 was performed to find diluted/adulterated specimens.

2.2.3 | Test Procedure 2 (TP2)—Novel Specimen Validity Testing Panel

TP2 includes the True Urine LD and True Urine SD Test from the VDx GEN2-Validity Panel. These two tests were installed and performed on an AU480 auto-analyser (Beckman Coulter, Krefeld, Germany) as described in the user manual and with consultation of the manufacturer. The assay principle is the target UTP in the authentic urine sample reacting with a chromogen generating reagent to form the UTP chromogen, which reacts in a second step with the colour developer to the UTP dye.

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According to the user manual, expected values for the LD test should be 12–1000 UTP-IV units (for females, 9–1000 UTP-IV units) with values < 12 or > 1000 UTP-IV units being invalid samples. According to the user manual, expected values for the SD test are 12–150 UTP II and III units (for females, 9–150 UTP II and III units), and SD values > 500 UTP II and III units should be considered suspicious. Based on the consultation of the manufacturer, SD values > 150 UTP II and III values are sometimes seen in authentic urine specimens. Thus, urine specimens were evaluated with three different acceptance criteria for the SD test: (1) 12–150 UTP II and III units, (2) 12–500 UTP II and III units and (3) ≥ 12 UTP II and III units. TP2 should be evaluated for its applicability to find SU among real-life urine specimens.

2.2.4 | Test Procedure 3 (TP3)—Established Advanced Specimen Validity Testing

TP3 includes two already established procedures for SU identification—(auto) analysis of UA and LC–MS/MS analysis of an EB pattern, including 10 previously described EB: phenylalanine, tryptophan, propionylcarnitine, butyrylcarnitine, isovalerylcarnitine, hexanonylcarnitine, phenylacetylglutamine, heptanoylcarnitine, octanoylcarnitine and indolacetylglutamine [13, 19]. UA (enzymatic detection, linearity of the test: 119–23,800 μmol/L, LOD = 10 μmol/L according to user manual) was analysed on an AU480 auto-analyser (Beckman Coulter, Krefeld, Germany). As for urea, there were no reference values for UA for single collected urine specimens found. In a previous study, UA concentrations were between 419 and 2822 μmol/L in single collected authentic urine specimens with CREA values ≥ 200 mg/mL [13]. Thus, UA analysis was evaluated as not passed when UA was not detectable and low when the UA concentration was < 419 μmol/L. Low UA values were compared to urea and CREA values for plausibility check. TP3 was used as reference method for evaluation of diagnostic sensitivity and diagnostic specificity of TP2 in this study.

2.2.5 | Sample Preparation for LC–MS Analysis

The preparation and measurement of US-SUs and real-life urine specimens by LC–MS/MS was the same as described in [13]. One hundred microliters of urine were mixed for 15 s with 500 μL of acetonitrile (ACN). Following 1 min centrifugation at 14,000 rpm, 500 μL of supernatant was transferred to autosampler vials. After gentle evaporation to dryness with nitrogen (60°C), the residue was resuspended in 50 μL of a 1:1, *v:v* mixture of mobile phases (MPs) A (10 mM aqueous ammonium formate, 0.1% formic acid) and B (ACN, 0.1% formic acid) [30]. For EB and direct SU marker detection, a LTQ XL linear ion trap coupled to a Dionex UltiMate 3000 ultra (high) performance LC system (Thermo Fisher Scientific, Dreieich, Germany) was used. Chromatographic separation was performed on a Nucleoshell RP 18 plus column (100 mm × 2.1 mm, 2.7 μm; Macherey-Nagel, Dueren, Germany) with the following gradient elution of MP A and B: 0–1.0 min 98% A, 1.0–3.0 min to 90% A, 3.0–5.0 min to 85% A, 5.0–7.5 min to 80% A, 7.5–10.0 min to 75% A, 10.0–11.5 min to 70% A, 11.5–13.0 min to 65% A, 13.0–14.5 min to 50% A, 14.5–16.0 min to 40% A, 16.0–19.0 min to 0% A, 19.0–20.0 min hold 0% A, 20.0–21.0 min to 98% A and 21.0–23.0 min hold 98% A [30]. The flow rate was set to 0.5 mL/min and the injection volume was 10 μL. The full-scan mode (FS, *m/z* 110–800) was used to acquire MS^1 data, and MS^2 as well as MS^3 spectra were obtained by data-dependent acquisition (DDA). MS and DDA parameters were adopted from Wissenbach et al. [30].

Low-resolution MS was used to detect both EB and the established previously described direct SU markers PPG, PPG + 16, and SUM255 [13, 19]. A urine sample was assigned to be of authentic origin if ≥ 6 EB and no direct SU markers were found.

2.2.6 | Detection of New Direct Synthetic Urine Markers

In order to unambiguously identify new direct SU markers, HRMS was performed on a Q Exactive Focus (Thermo Fisher Scientific, Dreieich, Germany) operating in full scan mode and simultaneously parallel reaction monitoring (PRM) mode. $HRMS^2$ spectra were obtained with a normalized collision energy of 50% and a resolution of 17,500. Careful $HRMS^2$ fragmentation pattern evaluation, reference spectra database search [31–34], and *in silico* prediction were used for presumptive identification of carbendazim, and reference material was used for final chromatographic and MS confirmation.

2.2.7 | Software

Data analysis of TP1 CREA, urea, UA and TP2 LD and SD markers was performed in Toxicom LIMS (2024.2.x). For LC–MS/MS handling, the Xcalibur software (4.0.2., Thermo Fisher Scientific) was used. The program ToxID (2.1.1., Thermo Fisher Scientific) coupled to the MWW reference library [32] was used for automatic EB assignment to DDA generated MS^2 spectra. This was confirmed by manual analysis of MS data in Xcalibur (4.0.2.), and established direct markers (PPG, PPG + 16) [13] were manually analysed in this way as well. For initial structural elucidation, mass spectra reference libraries [31–34] and the software Sirius (CSI:FingerID, Structure Database Search: all DBs, adduct: $[M+H]^+$, instrument: orbitrap, MS^2 mass accuracy: 10 ppm, MS/MS isotope scorer: ignore [35–38]) were used. Graphs and figures were generated in OriginPro 2024 and PowerPoint 2016, and chemical structures were made in ChemDraw 22.0.0. The graphical abstract was partially created with the free AI Image Generator—Image Creator in Bing.

3 | Results

3.1 | Authenticity Testing of Synthetic Urine Products

Two SU products from the US market were measured by TP1, TP2 and TP3 with the following results as given in Table 1.

With respect to TP1, US-SU1 had a CREA value of 520 mg/L and a urea value of 239 mmol/L and showed normal colour development on the test strips (CREA, nitrite, glutaraldehyde, pH, SG, oxidants/pyridinium chlorochromate). Thus, US-SU1 passed TP1. US-SU2 showed a CREA value of 480 mg/L and a urea

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TABLE 1 | Authenticity testing procedures and direct synthetic urine marker identification for two synthetic urine products from the US market.

	Indirect test												Direct test	
	Standard specimen validity testing (TP1)						VDx true urine LD/SD test (TP2)				Reference methods (TP3)				Direct markers	
Synthetic urine product	**Creatinine (mg/L)**		**Urea (mmol/L)**		**Surestep urine adulteration test strips**		**LD marker (UTP-IV units)**		**SD marker (UTP II and III units)**		**Uric acid (μmol/L)**		**LC-MS/MS EB**		**LC-MS/MS, LC-HRMS**	
US-SU1	520	✓	239	✓	Passed	✓	3	X	4	X	165	(X)[b]	0	X	PEG	X
US-SU2	480	✓	0	X	Passed	✓	3	X	3	X	0	X	0	X	Carbendazim	X
Acceptance criteria	≥ 200 [14, 15, 17] Linearity: 7.8–4200	✓	> 5.71 Linearity: 10–750	✓	According to colour scale	✓	12–1000	✓	12–150	✓	≥ 10 Linearity: 119–23,800	✓	≥ 6 [19]	✓	No detection	✓
Physiologic values	Females: 260–2170 Males: 390–2590 [15]		65–583 [13] 127 ± 25–698 ± 137[a] [39]		Creatinine [15] pH [14–17] Specific gravity [14–17] Absence of nitrite, glutaraldehyde and oxidants/pyridinium chlorochromate		Females: 9–1000 Males: 12–1000		Females: 9–150 Males: 12–150		419–2822 [13] 3.2 ± 1.1 mmol/1284 ± 468 mL[a] [40]		6–10 [19]		—	

Note: Given are the obtained values for the test parameters of test procedures (TP) 1, 2 and 3 with acceptance criteria and physiologic values. A tick indicates that the product passed the test, and a cross indicates that the product did not pass the test.
[a]Values obtained in 24h collected urine.
[b]Plausibility check with creatinine and urea failed.

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value of 0 mmol/L and passed the test strips. Thus, US-SU2 did not pass the urea measurement of TP1.

For TP2, US-SU1 had a value of 3 UTP-IV units with the LD test and 4 UTP II and III units with the SD test. US-SU2 had an LD marker value of 3 UTP-IV units and a SD marker value of 3 UTP II and III units. Thus, both US-SUs had LD/SD marker values < 12 UTP-IV/II and III units (lower limit for both tests according to the user manual) and did not pass the LD and SD test.

With respect to TP3, in US-SU1, a UA concentration of 165 μmol/L was determined, and no EB pattern was found by LC–MS/MS; in US-SU2, there was no UA, and no LC–MS/MS EB pattern detected.

3.2 | Authenticity Testing of Preselected Suspicious Specimen Set 1

Specimen set 1, including 47 preselected specimens (based on suspicious temperature and/or UA measurement), was used to evaluate the LD and SD test. Details for TP1, TP2 and TP3 are given in Figure 1 as well as in Table S1.

With TP1, five out of 47 specimens did not pass based on either low CREA values [14–17], no detectable urea and/or test strips indicating SG < 1.003 g/mL. In detail, in one specimen (No. 39), CREA was not detected; for the other 46 specimens, CREA values ranged from 50 (No. 10) to 2950 mg/L (No. 44) (mean = 794 mg/L). Urea was also not detectable in specimen No. 39, but detected in the other specimens, ranging from 17 (No. 10) to 433 mmol/L (No. 36) (mean = 230 mmol/L). The test strips showed for five specimens SG values < 1.003 g/mL according to the colour scale; the other 42 specimens passed all six reagent pads.

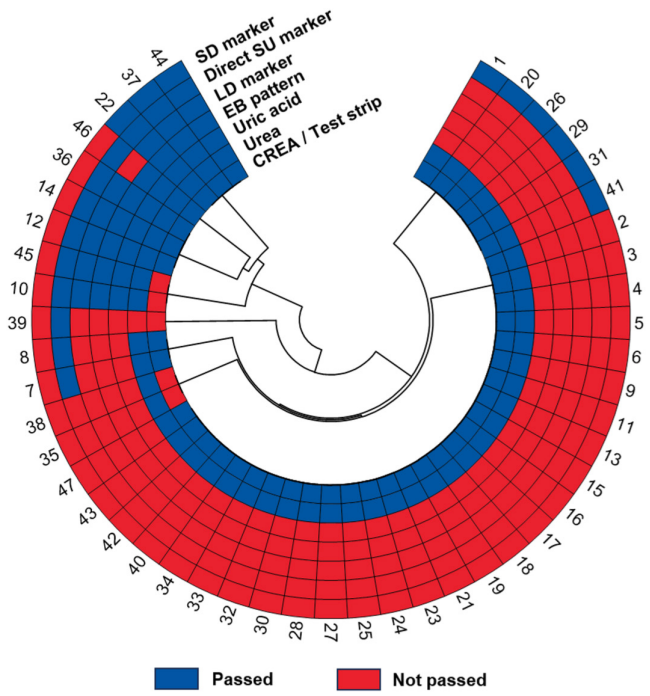

FIGURE 1 | Test details on passed (blue) and not passed (red) test parameters for the 47 preselected suspicious set 1 specimens.

The TP2 LD marker values were < 12 UTP-IV units (lower limit according to the user manual) in 39 out of 47 set 1 specimens. The range for the LD marker in the other eight specimens was 17 (No. 10) to 412 UTP-IV units (No. 44) (mean = 120 UTP-IV units). No specimen reached or exceeded the higher LD marker value limit of 1000 UTP-IV units. The TP2 SD marker was < 12 UTP II and III units (lower limit according to the user manual) in 37 out of 47 specimens. SD marker values of the other 10 specimens were between 12 (No. 44) and 187 UTP II and III units (No. 10) (mean = 42 UTP II and III units) with one specimen (No. 10) exceeding the higher limit of the normal range (150 UTP II and III units) of the SD test as given in the user manual.

With respect to TP3, 38 specimens did not pass UA analysis due to no detectable UA, whereas nine specimens passed with measured UA concentrations between 161 (No. 10) and 2822 μmol/L (No. 22) (mean = 1100 μmol/L). For TP3 LC–MS/MS indirect SU markers, < 6 EB detections out of 10 EB were found within the same 38 set 1 specimens as UA negatives. Precisely, in 36 of these 38 specimens, none of the EB targets was detected; in specimen No. 27, only phenylalanine was detected, and in specimen No. 7, only butyryl carnitine and phenylacetylglutamine were detected. Within the other nine set 1 specimens, EB patterns were detected by LC–MS/MS ranging from 7 EB (No. 10, 47) to 10 EB (Nos. 22, 36, 44) (mean = 8.6 EB LC–MS/MS detections).

3.2.1 | Sensitivity and Specificity of the True Urine LD and SD Test

TP3 UA and LC–MS/MS analysis of EB served as the gold-standard in this study to compare the TP2 LD and SD marker results to distinguish between authentic and substituted urine specimens. The congruency among LD and SD markers and UA/LC–MS/MS in evaluating the suspicious specimen set 1 is shown in Figure 2. Thirty-two specimens did not pass all four methods congruently. One specimen (No. 46) did not pass the TP2 LD and SD test but contained UA and LC–MS/MS EB and was therefore evaluated as authentic by TP3. Additionally, the SD test found four specimens with an SD marker value < 12 UTP II and III units. Respective false-positive rates (FPR) were 11.1% for the LD marker test and 56.6% for the SD marker test. Specimen No. 10, which exceeded the higher limit of 150 UTP II and III units (of the physiologic range according to the user manual), was excluded from the calculation, as TP1 (CREA = 50 mg/L, low SG) and TP3 (EB detections = 7) proved the dilution of an authentic specimen. Six of the 38 TP3 suspicious specimens passed the SD marker test with a respective false negative rate (FNR) of 15.8% for the SD marker. Thus, the LD marker had a diagnostic sensitivity of 100% and a diagnostic specificity of 88.9%, whereas the SD marker had a diagnostic sensitivity of 84.2% and a diagnostic specificity of 44.4%.

3.3 | False-Positive Rate of the True Urine SD Test in the Unsuspicious Specimen Set 2

UA was used to access again the FPR of the True Urine SD Test with the larger specimen set 2 ($n = 1141$). TP2 SD marker values were < 12 UTP II and III units in 190 set 2 specimens and ranged from 14 to 3775 UTP II and III units in the other 951 set 2 specimens. TP3 UA was detected in all set 2 specimens and

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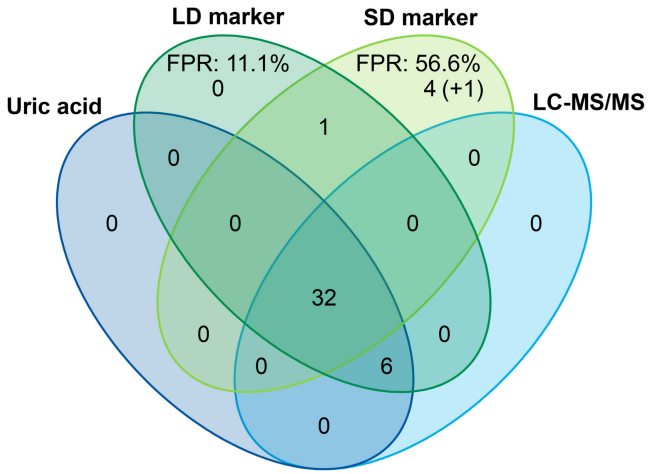

FIGURE 2 | Comparison of the True Urine LD Test, True Urine SD Test, uric acid analysis and LC-MS/MS EB pattern analysis for set 1 specimens. The VENN diagram shows four ellipses for the four compared test systems: True Urine LD Test (dark green), True Urine SD Test (light green), uric acid analysis (dark blue) and LC-MS/MS EB pattern analysis (turquoise). Not passed set 1 specimens by each method are given as numbers in the respective ellipse, while congruently not passed set 1 specimens by two or more methods are given as numbers in overlapping areas. Additionally, calculated false-positive rates (FPR) are shown for the True Urine LD and SD Tests.

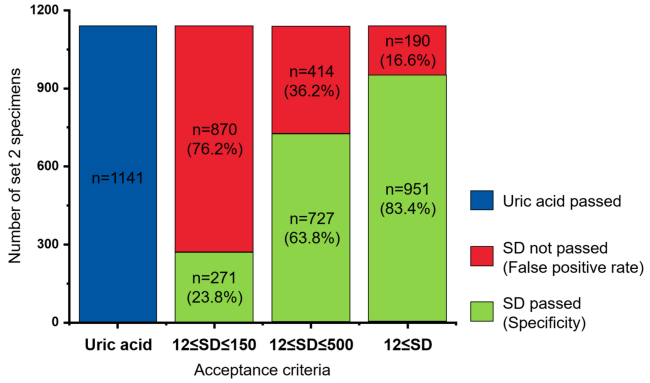

FIGURE 3 | Comparison of uric acid passed set 2 specimens (dark blue) with the True Urine SD Test results for three different applied acceptance criteria and respective diagnostic specificities (light green) and false-positive rates (red).

UA concentrations were between 199 and 10,862 µmol/L. There could be found no correlation between the SD marker value and UA. There was no correlation of the SD marker value to the CREA concentration, urea concentration and the LD marker value found as well.

Figure 3 illustrates the number of passed set 2 specimens for UA and the SD test with the respective acceptance criteria and FPR. Three different SD marker value acceptance criteria were applied: (1) 12 UTP II & III units as lower limit and 150 UTP II & III units as upper limit, (2) 12 UTP II & III units as lower limit and 500 UTP II & III units as upper limit, (3) 12 UTP II & III units as lower limit and no upper limit. For acceptance criteria (1), 271 set 2 specimens passed the SD test, whereas 870 set 2 specimens did not pass the SD test; here the FPR was 76.2%. For acceptance criteria (2), 727 set 2 specimens passed and 414 set 2 specimens did not pass with a FPR of 36.2%. For acceptance criteria (3), 951 set 2 specimens passed and 190 set 2 specimens did not pass the SD test; the calculated FPR with this acceptance criterion was 16.6%.

3.4 | LC–MS Analysis of Direct Synthetic Urine Markers

The SU products and the 47 set 1 specimens were analysed for direct SU markers. Detection of the established direct SU markers (PPG, PPG + 16, SUM255), which were described in [13, 19], was performed by LC–MS/MS in the same run as EB analysis. In US-SU1 and US-SU2, no PPG, PPG + 16, and SUM255 were detected. In US-SU1, however, PEGs were detected. With respect to specimen set 1, direct SU markers were detected in 35 out of 47 specimens, with simultaneous PPG and PPG + 16 detection in 29 specimens and solely PPG + 16 detection in six specimens. SUM255 was not found in any set 1 specimen.

3.4.1 | Identification of Carbendazim in Synthetic Urine

For identification of novel direct SU markers, LC-HRMS analysis was performed with US-SU1 and US-SU2 (Table 1). In US-SU2, a novel direct SU marker was detected at a retention time of 5.7 min, which was unambiguously identified by reference material as carbendazim ($C_9H_9N_3O_2$, $[M+H]^+ = 192.07675$, −0.224 ppm). The respective $HRMS^2$ spectra are shown in Figure S1 in the supporting information. Set 1 specimens were retrospectively analysed for carbendazim; it was, however, not detected in these 47 specimens.

4 | Discussion

Commercially available SU products have become a popular strategy to subvert urine drug testing as such SU products contain components (e.g. CREA, electrolytes) and present properties (pH, SG) as expected for authentic urine and produce negative drug screening results [8, 11–13]. Thus, standard SVT procedures [14–17] might not be sufficient in detecting manipulation attempts with SU [9, 13]. For this purpose, different methods were developed and evaluated to distinguish between synthetic and authentic urine specimens including test strip tests, auto-analyser based colorimetric tests, and chromatographic approaches [8, 9, 12, 13, 18, 19, 21–23]. While colorimetric tests have the advantage of being fast and cheap, chromatographic methods have longer analysis times and are more expensive. Nevertheless, chromatographic methods have the possibility to analyse the entirety of analytes contained in a sample and thereby can determine both indirect and direct SU markers in one measurement [9, 13].

This study aimed to evaluate the novel automatized assay True Urine LD and True Urine SD Test (TP2) from the VDx GEN2-Validity Panel [29] for revealing urine substitution especially by SU, which was to the author's knowledge the first study to

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evaluate this assay. Both assays have UTP as target analytes, which differ in their stability and are therefore named as 'long duration' (LD) UTP or 'short duration' (SD) UTP according to the user manual. While the LD test is claimed to uncover urine substitution by a different liquid, such as SU, the SD test is claimed to proof mishandling, adulteration or urine substitution. For a comprehensive evaluation of TP2, TP1, which included CREA, urea and Surestep Urine Adulteration Test Strip determination was performed. TP3, included two established procedures (UA and LC–MS/MS EB pattern analysis [13, 19]) in identifying urine substitution and was thus used as gold-standard in evaluating the new TP2.

Analysis of two SU products (Table 1) from the US market (US-SU1, US-SU2) with the TP2 LD and SD tests was able to detect urine substitution in both cases. TP3 UA was detected in low concentrations in US-SU1 (165 µmol/L) while TP1 CREA (520 mg/L) was in physiologic ranges according to Brenner-Hartmann [15] and TP1 urea (239 mmol/L) was evaluated as unsuspicious as well. To the authors knowledge, there are no reference values for both urea and UA in single collected urine specimens available. For medical issues, the urea/UA measurement is performed in 24 h collected urine of a patient. For urea, physiologic values are 342 ± 67 mmol in 490–2690 mL urine [39], which would be equal to a mean urinary urea concentration of 127 ± 25 mmol/L to 698 ± 137 mmol/L. A study on UA elimination using 24 h collected urine found 3.2 ± 1.1 mmol UA in 1284 ± 468 mL urine from 50 healthy persons [40], which would be a mean urinary UA concentration of 2492 µmol/L. Nevertheless, while UA was detected in this study in US-SU1 from the US market, UA was not found in SUs from the Austrian/German market [13]. This proves local differences in the SU composition, as Goggin *et al*. and Vikingsson *et al*. detected UA in some US-SU products in their studies [9, 12]. Nevertheless, as recently shown, UA analysis works for now in the Austrian/German area [13]. However, single substances such as UA may be easily added to SU products; thus, the market of available products should be constantly observed. In US-SU2, no UA and no urea were detected. With respect to TP3 LC–MS/MS EB analysis, none of the target analytes covering different compound classes were detected in the US-SUs, proving again its applicability in urine substitution testing [13, 19].

Real-life urine specimens ($n = 1188$) from drug testing were used to evaluate the novel TP2 LD and SD markers with TP1 and TP3 UA. Only a small proportion (specimen set 1) was further analysed by LC–MS/MS as urine substitution was indicated by suspicious temperature measurement and/or no detectable UA. Figure 1 and Table S1 (in the supporting information) provide detailed information on TP1, TP2, and TP3 results. TP2 LD and SD markers found 39 and 37 out of 47 set 1 specimens, respectively, undercutting the lower limits of 12 UTP-IV units and 12 UTP II & III units as given in the user manual, which were evaluated as not passed and thus substituted urine specimens. One specimen (No. 10, SD value = 187 UTP II & III units) exceeded the upper limit of 150 UTP II & III units of the SD test and was found to be a diluted authentic urine specimen by TP1 and TP3. Based on the consultation of the manufacturer, SD marker values >150 UTP II & III units are sometimes measured in authentic unsuspicious urine specimens. All set 1 specimens that did not pass TP2 or TP3 are shown in Figure 2. TP3 UA and LC–MS/MS EB analysis results were congruent for all 47 specimens and evaluated 38 set 1 specimens as substituted.

Based on TP3 results, the diagnostic sensitivity and specificity were calculated for TP2 LD and SD tests. The results of specimen set 1 indicated a very good performance of the True Urine LD Test (diagnostic sensitivity = 100%, diagnostic specificity = 88.9%, FPR = 11.1%) in finding substituted urine specimens (Figure 2). Thus, the LD marker might be a promising parameter to extend the standard SVT to reveal, besides dilution, chemical adulteration and substitution by another liquid, substitution by SU. For the True Urine SD Test, a lower performance was found in detecting substituted specimens (diagnostic sensitivity = 84.2%, diagnostic specificity = 44.4%, FNR = 15.8%, FPR = 56.6%). However, as the LD test also found one false positive (No. 46) further tests should be applied for confirmation. The user manuals of both tests advise performing the whole VDx GEN2-Validity Panel, which includes further tests and might increase the diagnostic specificity of the SD test. The present study only evaluated LD and SD markers of this panel, which is a drawback of this study.

For further evaluation of the SD test's FPR, the larger specimen set 2 ($n = 1141$), which consisted of temperature compliant and passed UA specimens from the Austrian/German region, was evaluated for SD test results. No confirmatory LC–MS/MS analysis was performed with this specimen set 2 because UA analysis still was found able to detect urine substitution by SU in the Austrian/German region, as shown in this and the previous study [13]. With different applied acceptance criteria for the SD test, it was shown that the FPR of the test was substantially influenced by the upper limit. Thus, when compared to specimen set 1, the FPR was even increased with acceptance criteria (1) as given by the user manual (12–150 UTP II and III units, FPR = 76.2%), decreased with acceptance criteria (2) following manufacturer consultation (12–500 UTP II and III units, FPR = 36.2%) and further reduced with acceptance criteria (3) without an upper limit for the SD value (FPR = 16.6%). However, since SD values > 500 UTP II and III units are indicated as substituted in the user manual, there should be an upper limit. Thus, a laboratory applying this SD test in urine authenticity testing should be aware of the varying probability to determine a specimen correctly authentic depending on the applied acceptance criteria of the SD test.

Besides the LD and SD tests described in this study also other automatized assays and test strips exist to uncover SU and urine adulteration [9, 13, 21–24]. The target analytes of such commercially available tests are often not declared by the manufacturer. For the LD and SD tests, proteins of the urinary tract are analysed, which have the advantage that adding them to SU products is less easy and cheap than adding salts or small molecules like CREA to SU. Additionally, preparation of specimens for an auto-analyser measurement is less elaborate, less time-consuming and less expensive than an MS-based approach. At least for the Austrian/German region, currently, detection of UA works to reveal urine substitution by a different liquid including SU [13]. In the United States, UA would only work for some products [9, 12]. Therefore, developing new, such as TP2, and advancing existing auto-analyser-based SU detection kits is needed. Nevertheless, an advantage of MS methods, like the

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applied LC–MS/MS method in this study, is the possibility to analyse EB patterns and direct SU markers simultaneously and is therefore a confirmatory method in identifying SU specimens compared to other approaches [9, 13, 19].

Thus, direct SU markers were investigated by LC–MS/MS as well in the two US-SUs and in the 47 set 1 specimens. The established PPG described by Kluge et al. [19] and PPG + 16 and SUM255 described in the previous study [13] were not detected in the two US-SU products. In US-SU1, however, PEGs were detected. Goggin et al. discussed the usage of PEGs as direct SU markers. Since PEGs were sometimes detected in German authentic urine specimens [19], they might not be sufficient in revealing SU usage for urine manipulation in the Austrian/German region. Nevertheless, in combination with the results from TP1 and LC–MS/MS EB pattern analysis, PEG presence could confirm this US-SU1 product as SU. In specimen set 1, PPG and PPG + 16 were detected in 35 of the 38 TP3 substituted specimens, identifying them as SU. Two of the three SU marker negative specimens were putatively SU as they passed TP1 and contained no EB pattern. Thus, there still is a gap in identifying some SU products, and further studies are needed to find also for these products direct markers for an unambiguous identification. The third SU marker negative specimen (No. 39) did not pass TP1 and was putatively a different liquid than SU.

A novel direct SU marker was found in US-SU2, which was at first presumptively identified by fragmentation pattern analysis (Figure S1), *in silico* prediction, and reference spectra databases and finally confirmed to be carbendazim by an analytical reference standard. Carbendazim is a benzimidazole fungicide usually used as a pesticide to protect crops [41]. It can only be speculated why it was found in the product, maybe as a preservative.

Overall, standard SVT parameters (TP1) including CREA, urea and adulteration testing (test strips), two new auto-analyser based assays targeting UTP (TP2) and established UA and LC–MS/MS EB pattern analysis (TP3) were applied to SUs from the US market and to real-life urine specimens. TP3 results were used for the evaluation of the TP2 diagnostic sensitivity and specificity. The True Urine LD Test showed a high diagnostic sensitivity and specificity in differentiating authentic from not authentic urine specimens. The True Urine SD Test had also a good diagnostic sensitivity, but diagnostic specificity substantially depended on the applied acceptance criteria with respect to the upper limit of the test. Additionally, LC-HRMS analysis was used to identify a novel direct SU marker, namely carbendazim, which was found in a US-SU.

5 | Conclusion

Several automatized assays were developed to be added to the standard SVT in order to detect substituted urine specimens by SU. In general, auto-analyser based techniques provide many advantages with respect to sample preparation effort, time and costs. At least in the Austrian/German region, automatized UA analysis proved again its reliability in detecting urine substitution. The results of this study indicated that the automatized LD marker analysis might advance the standard SVT to find substituted specimens including SU. The results of this study indicated for the SD marker a reduced diagnostic specificity which depended on the applied acceptance criteria and should be therefore interpreted in combination with other test parameters. As such test systems are of presumptive nature and target single endogenous analytes instead of larger EB patterns, they should be applied in combination with other confirmatory tests for verification. Manufacturers constantly improve and extend the constituent list of their SU products. Thus, it is important to screen new but also older SU products from time to time for endogenous urinary molecules. Therefore, LC–MS-based methods are most reliable to find newly added EB to SU products but also for identifying novel direct SU markers. Consequently, LC–MS is able to unambiguously differentiate between authentic urine and SU with the simultaneous analysis of indirect and direct SU markers.

Author Contributions

Laura Franke was involved in investigation, formal analysis, data interpretation, data visualisation and writing (original draft preparation). Christian Fuczik was involved in investigation, formal analysis and data interpretation. Dirk K. Wissenbach was involved in project administration, investigation and writing (review and editing). All authors critically revised and approved the article.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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