Comparison of Urine and Oral Fluid as Matrices for Screening of Thirty-Three Benzodiazepines and Benzodiazepine-like Substances using Immunoassay and LC–MS(–MS)

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Abstract

Benzodiazepines are the most frequently detected medicinal drugs in drivers. The use of benzodiazepines is associated with an increased road accident risk. In this study, the presence of benzodiazepines detected by liquid chromatography–(tandem) mass spectrometry [LC–MS(–MS)] in oral fluid and urine samples obtained from drivers stopped during a roadside survey was compared. In addition, the sensitivity and selectivity of enzyme multiplied immunoassay technique (EMIT® II Plus) relative to LC–MS(–MS) was determined for both matrices. A total number of 1011 urine samples were collected and screened for benzodiazepines using immunoassay (IA) (EMIT II Plus; cutoff 300 ng/mL). In the IA-positive (*n* = 25) and a group of randomly selected negative urine samples (*n* = 79), the presence or absence of benzodiazepines was confirmed by LC–MS–MS after deglucuronidation. The corresponding oral fluid samples (*n* = 101, 3 samples omitted), were analyzed by LC–MS(–MS) and IA (EMIT II Plus; cutoff 10 ng/mL). The presence of benzodiazepines was demonstrated by LC–MS–(MS) in all IA-positive urine samples, but in only four corresponding oral fluid samples. Concentrations in oral fluid were, one substance excepted, lower than in urine. The sensitivity and specificity of EMIT II Plus were better by using urine as matrix for screening of benzodiazepines than by using oral fluid. The results show that benzodiazepines are detectable in oral fluid. More research has to be done to determine the pharmacokinetic profile of the different benzodiazepines in oral fluid and to study the relationship between dose, concentration (in oral fluid and blood), and impairment.

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Introduction

According to article 8 of the Dutch Road Traffic Act, it is illegal to drive under the influence of alcohol or other psychoactive substances (1). In the Netherlands, the legal limit for alcohol (ethanol) is 0.5 mg/mL blood. However, there are no legal limits for other substances with a potential negative influence on driving performance. This makes the procedure for prosecuting drivers for driving under the influence of illegal and/or prescribed drugs rather complex (2).

The use of roadside test devices to examine illicit or medicinal drug use is not currently approved for judicial purposes in The Netherlands. If new legislation takes effect, sensitive and specific roadside tests will be required to detect the drivers who have used forbidden psychoactive substances. In several European countries, benzodiazepines are the most frequently detected psychoactive prescription drugs in drivers (3). The use of benzodiazepines has been associated with an increased risk of a motor vehicle crash (4,5). Currently, more than 20 different benzodiazepines and benzodiazepine-like substances can be prescribed by physicians (6). The commercial available roadside tests for the most frequently used illicit drugs and some classes of medicinal drugs (e.g., benzodiazepines) are immunoassay (IA) tests, testing for the presence of a group of chemical-related substances. These IA-tests do not have the

same sensitivity for all benzodiazepines, causing false-negative results for especially low-dose substances (7). Additionally, cross-reactivity with other medicinal drugs may lead to false-positive results.

One of the requirements for the implementation of roadside testing is the possibility to perform the test on samples that are relatively easy to obtain, like oral fluid or, to a lesser degree, urine. The advantage of urine as compared to blood is the longer period of traceability (8,9). The disadvantage is that parent substances or their (in)active metabolites may be longer present and detectable in urine than actual impairment exists. Oral fluid is regarded as the preferred specimen for roadside testing because of its low invasiveness of sampling and the better correlation with impairment as compared to urine (10). Oral fluid normally contains the parent compound (11), whereas urine also contains the metabolites. However, concentrations in oral fluid are lower than in urine. In this study, we compare the presence of benzodiazepines, metabolites and benzodiazepine-like substances in urine to oral fluid by using liquid chromatography–tandem mass spectrometry (LC–MS–MS). In addition, we compare the performance of immunoassay relative to LC–MS–MS, by using either urine or oral fluid as matrix for screening of benzodiazepines.

Methods

Setting and sample collection

This study was carried out in cooperation with the SWOV Institute for Road Safety Research (Leidschendam, The Netherlands), the Tilburg Police District, and the Dutch Laboratory on Drugs and Doping (Tilburg, The Netherlands). SWOV participates in the European research project, IMMORTAL (Impaired Motorists, Methods Of Roadside Testing and Assessment for Licensing) (12). IMMORTAL studies the examination of the prevalence of psychoactive substances (alcohol, illicit and medicinal drugs) in urine or serum samples of drivers and their effects on road injury risk.

For the present study (October 2002–March 2004) drivers were asked to provide an oral fluid sample on a voluntary basis in addition to a urine or serum sample. This allowed us to compare urine and oral fluid as matrix for roadside testing of benzodiazepines, their metabolites, and benzodiazepine-like substances. During the roadside survey, a mobile toilet was available. Samples were collected anonymously, and test results were not used for judicial purposes. Cooperation was rewarded with 5. Finally, the drivers were tested for alcohol by the police.

Urine samples were collected in plastic containers. Oral fluid samples were taken by chewing on a Salivette® (neutral cotton wool swab, Sarstedt, Germany) for 1 min. Specimens were collected within a few minutes. During the roadside sessions, urine samples were stored at 4°C, and oral fluid samples were stored in solid carbon dioxide at about –80°C (dry ice, Böhm & Gottschalk Droogijs BV, Netherlands). Data collection of the respondents included date and time of selection and self-reported drug and medicine use.

Procedure of analysis of the urine and oral fluid samples

In the laboratory, the Salivettes were thawed at room temperature and centrifuged (4000 rpm for 10 min). Until analysis, urine and oral fluid samples were stored at –20°C. A total of 1011 urine samples were collected and screened for benzodiazepines by using EMIT II Plus, using online deglucuronidation (13). The cutoff concentration for the benzodiazepine

Table I. Positive Results of the Analysis of Benzodiazepines in Urine and Oral Fluid using LC–MS(–MS)

	Validation Data Urine LC–MS–MS			Validation Data Oral Fluid LC–MS(–MS)						
Substance	LOD* (ng/mL)	LOQ† (ng/mL)	Recovery *n* = 6 (%)	LOD (ng/mL)	LOQ (ng/mL)	Recovery *n* = 6 (%)	Positive Urine Samples (*n*)	Concentration Range in Urine‡ (ng/mL)	Positive Oral Fluid Samples (*n*)	Concentration Range in Oral Fluid‡ (ng/mL)
Oxazepam	1.5	5.1	90	3.9	13.1	102	19	6–7028	2	18–1659
Temazepam	3.4	11.3	94	2.4	8.1	96	14	15–8074	0	< LOD
Nordazepam	1.4	4.7	80	2.3	7.6	88	4	51–166	0	< LOD
Midazolam	1.1	3.6	79	0.7	2.3	80	1	5	0	< LOD
OH-Midazolam	2.5	8.2	103	0.3	1.1	77	2	163–1815	0	< LOD
Alprazolam	0.7	2.2	69	0.3	1.2	100	4	27–78	3	5–9
OH-Alprazolam	0.5	1.6	73	0.2	0.5	83	5	6–279	0	< LOD
OH-Ethylflurazepam	0.2	0.6	68	0.8	2.7	85	1	153	0	< LOD
Lorazepam	0.3	1.1	76	0.3	1.2	89	1	14	0	< LOD
Lormetazepam	10.1	33.7	77	0.4	1.3	95	1	148	0	< LOD
Diazepam	0.0	0.0	73	2.1	6.9	84	1	4	0	< LOD
Zolpidem	1.4	4.9	66	3.0	10.0	89	1	7	1	51
Zopiclone	0.7	2.2	84	3.9	13.0	85	1	312	0	< LOD
Nitrazepam	0.4	1.2	76	0.0	0.1	98	1	30	0	< LOD

* LOD = limit of detection.
† LOQ = limit of quantification.
‡ Results are semiquantitative > 500 ng/mL (linearity was tested to 500 ng/mL).

assay was 300 ng/mL (14). In 25 urine samples (2.5%), the test result exceeded the cutoff value of 300. In those positive and in a group of randomly selected negative urine samples (n = 79), the presence of benzodiazepines, metabolites, and/or benzodiazepine-like substances was confirmed by LC–MS–MS. Before analysis by LC–MS–MS, the urine samples were hydrolyzed with β-glucuronidase/arylsulfatase (from Helix pomatia, about 30 U/mL β-glucuronidase and about 60 U/mL arylsulfatase, Merck, 12.5 µL/5 mL urine) overnight at about 37°C. In addition, the corresponding oral fluid samples, were analyzed by LC–MS(–MS) as well as EMIT II Plus (cutoff 10 ng/mL). In three cases, the volume of oral fluid collected was less than 100 µL; these were not analyzed. Therefore, the number of usable oral fluid samples, corresponding with the EMITII Plus positive urine samples (> 300 ng/mL), was 22.

Immunoassay

The screening method used in this study was enzyme multiplied immunoassay technique (EMIT II Plus) by using an ILab-600 immunoassay analyzer (Intrumentation Laboratory, Breda, The Netherlands). The EMITII Plus method was calibrated for lormetazepam in urine (0, 100, 200, 300, 1000 ng/mL, Dade Behring, Leusden, The Netherlands). The EMITII Plus method, which includes online deglucuronidation, has been validated for the analysis of urine samples (13). It was also used for the analysis of oral fluid samples, albeit without deglucuronidation, to study the performance of EMITII Plus in oral fluid. The oral fluid samples were automatically processed according to the Dade Behring protocol. Oral fluid used for the validation of the immunoassay was collected by chewing on a Salivette. Samples of different healthy volunteers were pooled after centrifugation of the Salivettes. Blank oral fluid samples were spiked with drugs by adding stock solutions in methanol, diluted with water. Validation of the assay for oral fluid samples included matrix interference, accuracy, and precision. Matrix interference was determined by analyzing 14 different blank oral fluid samples. Accuracy and intraday precision were estimated by analyzing oral fluid samples (n = 6) spiked with lormetazepam (10, 25, 50 ng/mL). The precision was expressed as the relative standard deviation. No external quality-control samples were available to estimate the accuracy of different benzodiazepines in oral fluid. The concentrations in oral fluid were calculated using calibration curves in urine. Cross-reactivity was estimated by analyzing two different oral fluid samples, spiked with one single substance: zolpidem (10, 100 ng/mL), oxazepam (10, 30, 50 ng/mL) or alprazolam (5, 10, 25 ng/mL). The immunoassays were performed at the Dutch Laboratory on Drugs and Doping (Tilburg, The Netherlands).

The matrix effects of oral fluid in the EMIT II Plus assay were acceptable at a cutoff concentration of 10 ng/mL (lormetazepam); the results of the blank oral fluid samples were between 0 and 8 ng/mL. The accuracy and precision were sufficient for the three concentration levels tested (n = 6): 10 ng/mL (mean 12 ng/mL; accuracy 121.7%; precision 6.2%), 25 ng/mL (mean 28 ng/mL; accuracy 113.3%; precision 3.6%), and 50 ng/mL lormetazepam (mean 52 ng/mL; accuracy 104.3%; precision 6.9%). Concerning the accuracy, a variation of 20% is generally considered acceptable (15).

The responses of the oral fluid samples spiked at 10 and 100 ng/mL zolpidem were 5 and 6 ng/mL, respectively, which are below the cutoff concentration of 10 ng/mL.

Cross-reactivity by using immunoassay was calculated as the mean response (n = 2) divided by the concentration of the substance and multiplied by 100. At the three concentration levels, oxazepam produced cross-reactivities of 160% (10 ng/mL), 203% (30 ng/mL), and 266% (50 ng/mL), respectively, compared to the calibrator substance lormetazepam. The percentage cross-reactivities of alprazolam were 280% (5 ng/mL), 230% (10 ng/mL), and 940% (25 ng/mL).

LC–MS(–MS)

The confirmation method used in this study was a quantitative analysis of 33 benzodiazepines, metabolites, and benzodiazepine-like substances, using APCI-LC–MS(–MS) (ion trap, Finnigan, The Netherlands) after matrix-supported liquid–liquid extraction (ChemElut® pH 9, Varian, The Netherlands). The method and the validation data for whole blood have been published previously (16). Sample volume was 1 mL. The method used an Xterra MS C-18 column (Waters) and a methanol/formic acid gradient (approximately 0.006M, pH 3, 30–60%, v/v, methanol). Because of the restrictions of the equipment in MS–MS mode concerning the number of scans per segments, MS detection was used for 14 substances and MS–MS detection for 19 substances. The injection volume was 50 µL. The LC–MS(–MS) method, originally developed for the analysis of benzodiazepines in whole blood, was validated for the analysis of urine and oral fluid after some minor modifications.

For the analysis of oral fluid, sample volume was reduced from 1.0 mL to 0.5 mL because of the limited volume of oral fluid. For the analysis of urine samples, MS–MS mode was demanded for all tested substances in order to reduce matrix interference and achieve lower limits of detection. Because of the limited number of scans per time-interval, two injections per urine sample were needed to screen for all benzodiazepines; consequently, the injection volume of urine was reduced to 25 µL. The analysis of urine and oral fluid samples by LC–MS(–MS) was performed at the Netherlands Forensic Institute (The Hague, The Netherlands).

Oral fluid and urine used for the validation of the method were obtained from healthy volunteers. Oral fluid was collected by having subjects chew on a Salivette. Samples of different subjects were pooled after centrifugation of the Salivettes. Blank oral fluid and urine samples were spiked with drugs by adding stock solutions in methanol diluted with water.

The method was found to be suitable for the analysis of the urine and oral fluid samples. For the substances detected in urine and oral fluid, the most important validation parameters in relation to the aim of this study [lower limit of detection (LOD), lower limit of quantification (LOQ), recovery] are presented in Table I.

Data analysis

Sensitivity, specificity, positive predictive value, and negative

Table II. Comparison of Urine, Oral Fluid, and Self-Reported Drug Use

Subject No.	Volume Oral Fluid (mL)	EMIT Urine* pos/neg	EMIT Oral Fluid† pos/neg	LC–MS–MS Urine‡ pos/neg	LC–MS–MS Oral Fluid‡ pos/neg	Urine Concentration (ng/mL)	Oral Fluid Concentration (ng/mL)	Self-Reported Drug Use
4609	1.25	p	n	p	n	Midazolam 5; OH-Midazolam 1815	–	–
4756	0.75	n	n	p	p	Alprazolam 27; OH-Alprazolam 22	Alprazolam 5	antidepressant 1–4 h before sampling
4810	0	p	–§	p	–§	Oxazepam 2894	–§	–
4813	1.25	p	n	p	n	Oxazepam 274	–	Oxazepam 12–24 h before sampling
4820	1.5	p	n	p	p	Oxazepam 76; Temazepam 337; Alprazolam 32; OH-Alprazolam 55	Alprazolam 6 –	Alprazolam 4–12 h before; Temazepam 12–24 h before sampling
4842	1.25	p	n	p	n	Oxazepam 1916	–	Oxazepam 20 mg 12–24 h before sampling
4919	1	p	p	p	p	Zopiclone 312; Alprazolam 78; OH-Alprazolam 279	Alprazolam 9	Alprazolam < 1 h before; Zopiclone 4–12 h before sampling
4938	1.75	p	p	p	n	Oxazepam 156; Temazepam 26; Nordazepam 51	–	Diazepam 12–24 h before sampling
4939	1.5	p	n	p	n	Oxazepam 276; Temazepam 1641	–	Temazepam 4–12 h before sampling
4954	0.75	p	n	p	n	Temazepam 168; Diazepam 4	–	–
4964	2	p	n	p	n	Oxazepam 2798; Temazepam 2798	–	Temazepam 10 mg 12–24 h before sampling
5011	1.75	p	n	p	n	Oxazepam 355; Temazepam 1196	–	Temazepam 10 mg 12–24 h before sampling
5217	0.1	p	–§	p	–§	OH-Midazolam 163	–§	Midazolam 7.5 mg 12–24 h before sampling
5301	0.5	p	n	p	n	Oxazepam 638; Temazepam 116; Nordazepam 166	–	–
5306	0.5	p	p	p	p	Oxazepam 7028; Zolpidem 7	Oxazepam 1659; zolpidem 51	hypnotic 4–12 h before sampling
5331	1	p	p	p	n	Oxazepam 6; Temazepam 24	–	Temazepam 4–12 h before sampling
5341	1.25	p	n	p	n	Oxazepam 4484	–	Oxazepam 4–12 h before sampling
5348	1.5	p	n	p	n	Oxazepam 607; Temazepam 5369	–	Temazepam 20 mg 4–12 h before sampling
5350	1	p	p	p	p	Oxazepam 2023; Temazepam 8074	Oxazepam 18	Temazepam 4–12 h before sampling
5634	1.5	p	n	p	n	OH-Alprazolam 6; Lorazepam 14; Lormetazepam 148	–	–
5764	1.5	p	n	p	n	Temazepam 15	–	–

* Cut-off value EMIT urine = 300 ng/mL.
† Cut-off value EMIT oral fluid = 10 ng/mL.
‡ Cut-off value LC–MS–MS urine and LC–MS(–MS) oral fluid = LOD (see Table I).
§ No oral fluid sample available.

Table II. (Continued) Comparison of Urine, Oral Fluid, and Self-Reported Drug Use

Subject No.	Volume Oral Fluid (mL)	EMIT Urine* pos/neg	EMIT Oral Fluid† pos/neg	LC–MS–MS Urine‡ pos/neg	LC–MS(–MS) Oral Fluid‡ pos/neg	Urine Concentration (ng/mL)	Oral Fluid Concentration (ng/mL)	Self-Reported Drug Use
5777	1.5	p	n	p	n	Oxazepam 243; Temazepam 73; Nordazepam 103	– –	Temazepam 10 mg 4–12 h before sampling
6028	0.75	p	p	p	n	Oxazepam 53; Temazepam 537; OH-Ethylflurazepam 153	– –	Temazepam 20 mg 12–24 h before sampling
6052	1.25	n	n	p	n	Nitrazepam 30	–	–
6058	0	p	–§	p	–§	Alprazolam 28; OH-Alprazolam 17	–§	–
6143	2.25	p	n	p	n	Oxazepam 78; Temazepam 78; Nordazepam 77	– –	Diazepam 5 mg 4–12 h before sampling
6161	1.25	p	n	p	n	Oxazepam 6559	–	–

* Cut-off value EMIT urine = 300 ng/mL.
† Cut-off value EMIT oral fluid = 10 ng/mL.
‡ Cut-off value LC–MS–MS urine and LC–MS(–MS) oral fluid = LOD (see Table I).
§ No oral fluid sample available.

predictive value of the IA compared to LC–MS–MS were calculated according to the formulas:

Sensitivity = True Positives/(True Positives + False Negatives)

Specificity = True Negatives/(True Negatives + False Positives)

Positive predictive value = True Positives/(True Positives + False Positives)

Negative predictive value = True Negatives/(True Negatives + False Negatives)

Analytical results were compared with self-reported drug use.

Results

Benzodiazepines analyzed using LC–MS(–MS)

Table I shows the substances detected in urine and oral fluid (n total = 102) by using LC–MS(–MS), the most relevant validation data in relation to this study (LOD, LOQ, recovery), the number of positive urine and oral fluid samples and the concentration (range) in urine and oral fluid.

One urine sample, with an IA test result of 256 (i.e., below the cutoff), was also analyzed by LC–MS–MS because of self reported drug use.

The samples of the control group, consisting of 79 urine samples (IA result < 300 ng/mL) and the corresponding oral fluid samples, were all negative for benzodiazepines, metabolites, and/or benzodiazepine-like substances by LC–MS(–MS), one urine sample excepted. In this sample, nitrazepam was detected at a concentration of 30 ng/mL.

The most frequently detected benzodiazepines in the collected urine samples are oxazepam (n = 19) and temazepam (n = 14). Detected substances in oral fluid are oxazepam (n = 2), alprazolam (n = 3), and zolpidem (n = 1).

Substances and concentrations detected in urine and oral fluid

Table II presents the individual analytical results of the positive urine and/or oral fluid samples (n = 27). The substances detected in urine are mentioned and compared to the substances detected in the corresponding oral fluid samples, which allows us to compare the presence and the concentrations of the benzodiazepines, metabolites, and benzodiazepine-like substances. Table II includes the cases with a positive IA result in urine (> 300 ng/mL; n = 25), the case with an IA result of 256 in urine (n = 1; subject no. 4756) and the control with a positive result in urine by using LC–MS–MS (n = 1; subject no 6052). Additionally, self-reported drug use is mentioned.

In all urine samples, with a positive result by using IA (n = 25), the presence of benzodiazepines was confirmed by using LC–MS–MS. The volume of three corresponding oral fluid samples was less than 100 µL. In only four of the remaining oral fluid samples (n = 22), the presence of a related substance was detected by using LC–MS(–MS). In the urine sample with an IA result of 256, the presence of alprazolam and its metabolite OH-alprazolam were confirmed. In the corresponding oral fluid sample, alprazolam was found. Concentrations of alprazolam and oxazepam in oral fluid were lower than concentrations in the corresponding urine samples. The oral fluid/urine (OF/U) ratios for alprazolam were 0.18 (5:27), 0.19 (6:76), and 0.12 (9:78). The OF/U ratios for oxazepam were 0.24 (1659:7028) and 0.009 (18:2023). The concentration zolpidem in oral fluid was higher than the concentration in urine. The OF/U ratio was 7.28 (51:7).

Immunoassay compared to LC–MS(–MS)

Tables III and IV show the sensitivity and specificity for EMIT II Plus compared to LC–MS(–MS).

The sensitivity, specificity, and positive predictive value of EMIT II Plus (n total = 101) were higher by using urine as ma-

Table III. Sensitivity and Selectivity for EMIT II Plus Compared with LC–MS–MS for Urine*

	LC–MS–MS Urine		
EMIT II Plus Urine	Positive	Negative	Total
Positive	22	0	22
Negative	1	78	79
Total	23	78	101

* Sensitivity = 95.7%, Specificity = 100%, Positive Predictive Value = 100%, Negative Predictive Value = 98.7%.

Table IV. Sensitivity and Selectivity for EMIT II Plus compared with LC–MS(–MS) for Oral Fluid*

	LC–MS(–MS) Oral Fluid		
EMIT II Plus Oral Fluid	Positive	Negative	Total
Positive	3	3	6
Negative	1	94	95
Total	4	97	101

* Sensitivity = 75%, Specificity = 96.9%, Positive Predictive Value = 50%, Negative Predictive Value = 98.9%.

trix (cut-off 300 ng/mL) than by using oral fluid (cut-off 10 ng/mL). The negative predictive values were almost equal for urine (98.7%) and oral fluid (98.9%). In three cases (Table II, subject nos. 4938, 5331, and 6028) the immunoassay in oral fluid showed a false-positive result; the presence of benzodiazepines in oral fluid was not confirmed with LC–MS–MS.

Discussion

In 25 of the 1011 urine samples from drivers stopped during the roadside survey, the presence of benzodiazepines was indicated by immunoassay and confirmed using LC–MS–MS. Sample collection took place on-site. Analysis of the samples by immunoassay and LC–MS–MS was performed in the laboratory. In only four corresponding oral fluid samples, the presence of a benzodiazepine was detected by using LC–MS–MS. The performance of the immunoassay was better by using urine after deglucuronidation than by using oral fluid as matrix.

The prevalence of benzodiazepines in our study population (2.5%) is comparable to the percentage of benzodiazepine-positive samples collected from drivers stopped during a roadside survey (1.5%) reported by Movig et al. (17). Our results are in agreement with the findings that oral fluid contains mainly the parent compound; in three oral fluid samples we confirmed the presence of alprazolam. In the corresponding urine samples alprazolam and its metabolite OH-alprazolam were detected. This may be explained by the generally higher hydrophilicity of metabolites, which promotes their excretion in urine.

Concentrations of alprazolam and oxazepam were lower in oral fluid than in the corresponding urine samples. The oral fluid/plasma ratio of benzodiazepines is already expected to be less than unity, because benzodiazepines and their active metabolites bind to plasma proteins and the concentration of benzodiazepines in oral fluid is expected to represent only the unbound fraction in blood. The extent of binding ranges from about 70% for alprazolam to nearly 99% for diazepam (18). As a result, the saliva/plasma ratio of benzodiazepines ranges from 0.01 to 0.082 (8).

Unknown is whether the subjects in this study were chronic users or not and whether chronic use will lead to an accumulation of drug in oral fluid or not. The oral fluid/urine ratio is influenced by the extent of metabolism. In one subject, the concentration of zolpidem was higher in oral fluid (51 ng/mL) than in urine (7 ng/mL). This can be explained by the fact that zolpidem is converted to inactive metabolites and less than 1% of an administered dose is excreted unchanged in the urine (19). Oral fluid is expected to test positive for zolpidem for over 8 h (20).

The relatively small number of oral fluid samples with a positive test result for benzodiazepines, in relation to the number of positive urine samples indicates a shorter detection time in oral fluid than in urine. To our knowledge, data about the windows of detection of benzodiazepines in oral fluid are limited (21–23). Most studies performed in the 1980s or earlier involved the analysis of specific substances after intake of a defined dose using very sensitive but less specific methods than LC–MS–MS. The use of LC–MS–MS allowed us to screen for benzodiazepines. The LODs for benzodiazepines in oral fluid are low and will meet the criteria for screening purposes; but to be able to detect all benzodiazepines in lower concentrations, a more sensitive method might be needed.

In cases of (self-reported) recent use of drugs, relatively high concentrations in oral fluid are expected. However, the number of positive oral fluid samples was lower than the number of recent drug use self-reports (i.e., 4–12 h before sampling). Unfortunately, in this study, data from the medication records of the subject were not available to support the reported drug use. The analytical results in urine correspond well with self-reported drug use. It is unknown whether this would be the case if the information were to be used for judicial procedures. Comparison of the screening results in urine and oral fluid with blood concentrations would have given valuable additional information. The number of positive blood samples is expected to be higher than the number of positive oral fluid samples because of the lower concentrations in oral fluid compared to blood. Unfortunately, in this study, blood sampling of all drivers was not possible.

Concerning the concentrations of the benzodiazepines in oral fluid, no correction for the recovery of the different benzodiazepines by using the Salivette for oral fluid collection was made, although it is known that the collection device might influence the measured concentration in oral fluid (24).

Before introducing a roadside screening for forensic purposes, it is meaningful to test the procedure in a laboratory setting to get more insight in the results to be expected on-site. Although the samples were analyzed in the laboratory, sample collection took place on-site.

The immunoassay we used for the analysis of the oral fluid samples was, according to the manufacturer's instructions, developed for the analysis of urine samples. The sensitivity and specificity of the immunoassay were higher by using urine as matrix than by using oral fluid. Our results support the finding that the requirements of a screening method for oral fluid are different from the requirements of an immunoassay for screening in urine (25). Although the validation characteristics seemed to be satisfactory, the influence of several factors (e.g., pH, protein binding, and matrix components) on the performance of the immunoassay in oral fluid is unknown. Another important factor is the choice of the cutoff value for benzodiazepines. A lower cutoff value will result in higher sensitivity but will also lead to more false positive results and consequently lower specificity. The cutoff value depends on the goal of the investigation and may be different for workplace testing, therapeutic monitoring, or driving under the influence. Therefore, the development of international guidelines concerning cutoff values for driving under the influence is recommended.

High sensitivity is required for the detection of benzodiazepines in oral fluid, in which drug concentrations are lower than in urine. To reduce the effect of potential interfering substances in the matrix, a separation stage prior to detection could be necessary. The antibodies should be directed to the parent drug in case of screening in oral fluid. Another important feature is the cross-reactivity. Benzodiazepines show cross-reactivity to the calibrator substance; the extent of cross-reactivity is depending on the substance as well as the concentration tested. Information about cross-reactivity of the benzodiazepines at several concentrations may be helpful to explain false-positive or false-negative test results.

In this study, the presence of other substances than benzodiazepines, metabolites, and related substances was not analyzed. Other substances could have influenced the performance of the immunoassay or could have influenced the concentrations in oral fluid because of interference with the metabolism of benzodiazepines.

The commercially available roadside tests for illicit drugs and benzodiazepines are under evaluation in the EU/US Project ROSITA-2 (Roadside Testing Assessment) (26). More research has to be done to establish the pharmacokinetic profile of the different benzodiazepines and related substances in oral fluid in relation to blood. In the Netherlands, the most frequently detected benzodiazepines in blood samples of drivers are diazepam, nordiazepam, temazepam, and oxazepam (2). Based on the relatively high blood concentrations, those substances are also expected to be detectable in oral fluid. More data about the window of detection in oral fluid are necessary to be able to advice about the period not to drive after intake of the medication. Another important question remains whether there is, and if so how to demonstrate, a relationship between the concentration of benzodiazepines (in oral fluid and in blood) and impairment. Do the available roadside tests meet the criteria to detect the concentrations of benzodiazepines in oral fluid which are relevant in relation to impairment? More research has to be done to study the dose-impairment relationship of benzodiazepines.

According to proposed new Dutch legislation concerning driving under the influence of other substances than alcohol, in a first step, the police officer will have to determine if there is a suspicion of impaired driving ability using four standardized behavioral tests. In cases of suspicion, a biological sample will be collected for a roadside drug test. In case of a positive roadside test, the presence of the substance will have to be confirmed in blood by a specific method in order to serve as evidence in court. Because of the large number of different benzodiazepines and the limitations of an immunoassay test (i.e., cross-reactivity, low-dose and high-dose benzodiazepines), the test result only gives an indication of the use of benzodiazepines. The sensitivity and specificity of the commercially available immunoassay test will determine the value of the test in roadside procedures. Policy makers should be aware of false-positive and false-negative test results.

Conclusions

Benzodiazepines were detected in oral fluid samples, collected during road side surveys. Oral fluid testing may be promising, because at the roadside, oral fluid was found to be easier to collect than urine. Concentrations in oral fluid were, one substance excepted, lower than in urine. The performance of EMIT II Plus was better when using urine as matrix for screening of benzodiazepines than when using oral fluid. The analytical results in urine correspond well with self-reported drug use. More research has to be done to determine the pharmacokinetic profile of the different benzodiazepines in oral fluid and to study the relationship between dose, concentration (in oral fluid and blood), and impairment.

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