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Direct urine analysis for the identification and quantification of selected benzodiazepines for toxicology screening

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ABSTRACT

A simple and rapid LC/MS method with direct injection analysis was developed and validated for the identification and quantification of ten benzodiazepines (flunitrazepam, nordiazepam, diazepam, 7-aminoflunitrazepam, flurazepam, bromazepam, midazolam, alprazolam, temazepam and oxazepam) in human urine using diazepam-d5 as internal standard (IS). The main advantage of the proposed methodology is the minimal sample preparation procedure, as diluted urine samples were directly injected into LC/MS system. Electrospray ionization in positive mode using selected ion monitoring was chosen for the identification and quantification of the analytes. The linear range was 50–1000 ng/mL for each analyte, with square correlation coefficient (r^2) ≥ 0.981. Interday and intraday errors were found to be ≤5.72%. The LC/MS method was applied at ten real samples found initially to be positive and negative, using immunoassay technique. Finally the results were confirmed with GC/MS. The method demonstrates simplicity and fast sample preparation, accuracy and specificity of the analytes which make it suitable for replacement of immunoassay screening in urine avoiding thus false negative/false positive results. Using this method, laboratories may overcome the problem of high cost instrumentation such as LC–MS/MS by providing similar sensitivity and specificity with other methods.

1. Introduction

Benzodiazepines are widely prescribed as anxiolytics, sedative hypnotics, anticonvulsants or muscle relaxants [1,2]. Clinical popularity of benzodiazepines has been ascribed to the wide safety margin of their therapeutic index, minimal serious adverse side-effects and low potential for physical dependence [3]. Apart from their therapeutic applications, benzodiazepines are often abused by drug addicts. As a consequence, these drugs are frequently involved in both clinical and forensic cases [2].

A number of studies have been reported in the literature on the determination of benzodiazepines and their metabolites in biological fluids. These studies use either immunological methods [4–6] or chromatographic techniques [7–13]. Enzyme immunoassay assays (EIAs) are known to provide rapid results for preliminary screening in urine for the presence of benzodiazepines with usual cut-off level of 100 ng/mL; however, specificity remains a significant disadvantage of such methods [14] where this class of drugs appears to cross react with other substances in a number of cases [15–17]. In one study, 16 of 50 positive benzodiazepine EIAs were submitted by patients taking sertraline who were not ingesting benzodiazepines. In a review phase of the same study, false-positive benzodiazepine EIAs caused by sertraline were found in 26% of 2447 patients taking sertraline urine specimens [17].

Gas chromatography, coupled with mass spectrometry, is not applicable to the determination of the entire range of benzodiazepine panel, because of the thermal instability or scarce volatility shown by some of them, even after derivatization [18]. In contrast, methods based on liquid chromatography (LC) hyphenated with mass spectrometry (MS) are successfully employed for all benzodiazepines [19].

LC–MS/MS appears as the most eligible technique for the simultaneous determination of several benzodiazepines [20]. However, and despite the increased use of LC–MS/MS instrumentation in toxicological laboratories, the high cost of the instrument remains a main problem that make it not always available. Most of the reported LC–MS/MS methods [1,21,11,22,19,23,24] in the literature appear to use traditional solid phase (SP) or liquid–liquid (LL) extraction techniques which both require costly consumables such as cartridges and solvents. Two other articles present the determination of selected benzodiazepines using column switching [25] and on line extraction [26] that require particular system configuration which are not always available in a toxicological laboratory. Additionally, de Jager and Bailey [26] determined only a small

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number of benzodiazepines, used time consuming glucuronidation procedure and did not evaluate the method in authentic samples. Time consuming glucuronidation procedure in combination with liquid–liquid extraction or protein precipitation were also used by Salomone et al. [27] and Ming and Heathcote [28], respectively, while the LOQ values do not significantly differ [27] as they are proposed in this article.

The aim of the present work was to develop a direct injection liquid chromatography–single quadruple mass spectrometric method for the separation, identification and quantification of 11 benzodiazepines (diazepam, nordiazepam, temazepam, oxazepam, flunitrazepam, 7-amino-flunitrazepam bromazepam, flurazepam, clobazam, midazolam, alprazolam) in urine, some of which are metabolites of others.

Using the direct injection technique, the method provides the advantage of a much simpler and faster sample preparation than the proposed in the literature techniques of SP, LL, column switching and on line extraction. Furthermore, direct injection procedure is completed in very short time. Finally, the method provides the advantage of specificity with lower cut-off levels comparing to immunoassay methods while the cost of a filter per sample is lower than the respective cost of the immunoassay kit per sample. Weakness of the method appears to be the run time of 40 min comparing to an immunoassay method. This is because the authors preferred to avoid the increased matrix effect occurring in single quadruple mass spectrometer at the beginning of the run, which was then significantly decreased after 15 min, increasing thus the sensitivity of the method. In that way, a single MS detector is demonstrated to be sufficiently applicable for benzodiazepines screening, without the use of high cost instrumentation such as LC–MS/MS.

The method was fully validated and successfully applied for the investigation of specific cases of clinical and forensic interest and is proposed for screening purposes.

2. Materials and methods

2.1. Chemicals and reagents

Reference standards of 10 benzodiazepines and diazepam-d5 (internal standard (IS)) were obtained from LGC Promochem (Molsheim, France). Acetonitrile was of HPLC grade and purchased from Merck (Darmstadt, Germany). Water was deionized and further purified by means of a Direct-Q water purification system from Millipore SA (Molsheim, France). Formic acid was of analytical grade and was obtained also from Merck. Millex-HV 0.45 μm syringe-driven filters were obtained also from Millipore SA. Emit II Plus Benzodiazepine Assay kit was obtained by Siemens Healthcare Diagnostics Inc. (Newark, DE, USA).

2.2. Liquid chromatographic conditions

A Shimadzu LC system (Shimadzu, Kyoto, Japan) consisting of a LC 20AB pump equipped with a DGU $20A_5$ degasser and a SIL 20AC autosampler, was used for the chromatographic separation. Chromatographic separations were achieved on a XTerra® MS C8, analytical column (250 mm × 2.1 mm i.d., 5 μm; Waters Corporation, Milford, MA, USA). The elution solvents were 0.05% formic acid (solvent A) and acetonitrile (solvent B). The mobile phase eluted under the following linear gradient conditions: (a:b; v/v) from 1:99 to 25:75 in 15 min, fast gradient to 1:99 in 15.01 min and then isocratic elution until 40.0 min for re-equilibration. The flow rate was stable (0.25 mL/min) for the first 5 min, increased to 0.4 mL/min from 5.01 min to 25 min and return to 0.25 mL/min for re-equilibration. The analysis run time was 40 min and the injection volume was 10 μL. The data were acquired and processed using LCMSsolution software (version 3, Shimadzu).

Table 1
$[M+H]^+$ ions and retention time used for the detection of the selected benzodiazepines in human urine.

Benzodiazepine	*m/z*	R_t (min)
Nordiazepam	271	18.9
7-Aminflunitrazepam	284.3	16.3
Diazepam	285	20.1
Oxazepam	287	18.7
Temazepam	300.9	19.5
Alprazolam	309	19.0
Flunitrazepam	314	19.6
Bromazepam	317.8	17.4
Midazolam	326.7	16.3
Flurazepam	388	16.4

2.3. Mass spectrometric conditions

The mass spectrometer was a single quadruple mass spectrometer (Shimadzu, LCMS 2010EV, Kyoto, Japan) with an electrospray ionization (ESI) source operating in positive mode. Nitrogen was used as drying and nebulizer gas and the instrument parameters were optimized by tuning the $[M+H]^+$ ions of the analytes, by direct infusion of their standard solutions. The Nebulizer gas flow was set to 1.5 L/min. CDL and Heat Block temperatures were optimized and kept at 230 °C. The appropriate CDL, Q-array DC and Q-array RF voltages were adjusted to 3, 5, 5, and 140 V, respectively. The selected benzodiazepines were detected using $[M+H]^+$ ions and their mean retention times were recorded according to Table 1.

2.4. Stock and working standard solutions

A mixed working standard solution of flunitrazepam, nordiazepam, diazepam, 7-aminoflunitrazepam, flurazepam, bromazepam, midazolam, alprazolam, temazepam and oxazepam at 10 μg/mL was prepared from their appropriate separate stock solutions of 1 mg/mL. This mixture was then used for sample spiking to provide calibration curves in urine. Flunitrazepam, nordiazepam, diazepam, 7-aminoflunitrazepam, flurazepam, bromazepam, midazolam, alprazolam, temazepam and oxazepam working solutions in the calibration range 0.5–10 μg/mL were prepared by dilution of the 10 μg/mL mixed standard solution with ultrapure water. Quality control (QC) samples were prepared by appropriate dilutions using separate stock solutions of different batch to obtain final concentrations of 1.5, 5 and 8.5 μg/mL. Working standard solution of IS was prepared at 1000 ng/mL by dilution of 10 μg/mL standard solution with ultrapure water.

2.5. Calibrator and quality control samples

Urine standards for calibration curves and QC were prepared by spiking 0.9 mL aliquots of drug free human urine with 100 μL of the appropriate mixed standard solution of benzodiazepines, providing a calibration range between above 50–1000 ng/mL and QC levels at 150–500–850 ng/mL.

2.6. Validation procedure

Spiked human urine calibrator at seven concentration levels, were prepared and analyzed in five different analytical runs. Calibration curves on the basis of peak area ratio of the ten benzodiazepines to that of the IS versus the theoretical concentration were prepared for each run. Weight ($1/x^2$) least square linear regression was used to obtain the equations of the calibration curves.

Table 2
Linearity results, LOD and LOQ of the proposed method for the selected benzodiazepines in human urine.

Benzodiazepine	Regression equation[a]	r^2[b]	LOD[c]	LOQ[d]	S_r[e]
Nordiazepam	$R_{ndz} = 2.763 \times 10^{-3} (\pm 1.0 \times 10^{-4}) C_{ndz} + 2.35 \times 10^{-1} (\pm 9.0 \times 10^{-3})$	0.997	10.75	32.57	1.429×10^{-4}
7-Aminflunitrazepam	$R_{amnf} = 3.612 \times 10^{-3} (\pm 1.0 \times 10^{-4}) C_{amnf} + 1.79 \times 10^{-1} (\pm 1.5 \times 10^{-2})$	0.991	13.70	41.53	3.272×10^{-4}
Diazepam	$R_{dz} = 4.154 \times 10^{-3} (\pm 1.0 \times 10^{-4}) C_{dz} + 5.223 \times 10^{-2} (\pm 1.7 \times 10^{-2})$	0.993	13.51	40.92	2.988×10^{-4}
Oxazepam	$R_{oxz} = 9.527 \times 10^{-4} (\pm 1.0 \times 10^{-4}) C_{oxz} - 4.244 \times 10^{-2} (\pm 4.0 \times 10^{-3})$	0.991	13.86	41.98	7.877×10^{-5}
Temazepam	$R_{tmz} = 8.681 \times 10^{-4} (\pm 1.0 \times 10^{-4}) C_{tmz} + 9.311 \times 10^{-3} (\pm 4.0 \times 10^{-3})$	0.994	15.20	46.08	6.041×10^{-5}
Alprazolam	$R_{aprz} = 3.282 \times 10^{-3} (\pm 1.0 \times 10^{-4}) C_{aprz} + 1.66 \times 10^{-1} (\pm 1.4 \times 10^{-2})$	0.993	14.08	42.66	2.452×10^{-4}
Flunitrazepam	$R_{fltz} = 8.745 \times 10^{-4} (\pm 1.0 \times 10^{-4}) C_{fltz} - 8.377 \times 10^{-3} (\pm 4.0 \times 10^{-3})$	0.992	15.09	45.74	6.945×10^{-5}
Bromazepam	$R_{brmz} = 1.057 \times 10^{-3} (\pm 1.0 \times 10^{-4}) C_{brmz} + 1.564 \times 10^{-3} (5.0 \times 10^{-3})$	0.987	15.61	47.30	1.059×10^{-4}
Midazolam	$R_{mdz} = 4.350 \times 10^{-4} (\pm 1.0 \times 10^{-4}) C_{mdz} + 1.085 \times 10^{-2} (\pm 2.0 \times 10^{-3})$	0.981	15.17	45.98	5.236×10^{-5}
Flurazepam	$R_{flz} = 1.285 \times 10^{-3} (\pm 1.0 \times 10^{-4}) C_{flz} + 1.199 \times 10^{-2} (\pm 4.0 \times 10^{-3})$	0.997	10.27	31.13	6.064×10^{-5}

[a] Ratio of the peak area of the selected benzodiazepines to that of the *IS*, *R*, versus the corresponding concentration *C*.
[b] Square correlation coefficient of the calibration curves.
[c] LOD as calculated according to the equation $3.3 \times S_b/a$ (ng/mL); S_b corresponds to the standard deviation of intercept; a corresponds to the slope of the calibration curve.
[d] LOQ as calculated according to $10 \times S_b/a$ (ng/mL); S_b corresponds to the standard deviation of intercept; *a* corresponds to the slope of the calibration curve.
[e] Standard error of the estimate.

QC samples were processed in six replicates at each concentration for five different analytical runs in order to evaluate the intra- and inter-assay precision and accuracy. Precision was assessed as the percentage relative standard deviation (%RSD) for each concentration level. Accuracy was defined as the relative difference between the calculated and theoretical concentrations of the ten benzodiazepines.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated according to signal/noise (S/N) ratio and from the equations of the calibration curves.

Specificity was checked by analyzing human urine samples spiked with drugs that could be found in a real samples (cocaine, ecgonine methylester, benzoyloecgonine, morphine, codeine, 6-acetyl-morphine, Δ^9-tetrahydrocannabinol, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, methadone, phenobarbital, clozapine, carbamazepine, venlafaxine, amphetamine, methamphetamine, paroxetine, citalopram, clomipramine, mirtazapine, amitriptyline, biperiden, ephedrine, ketamine, phenytoin, MDMA, haloperidol and thioridazine). Concentrations in these mixtures were 1 μg/mL. In addition, six different blank human urine were analyzed in order to evaluate the absence of interfering peaks in the ion chromatographs of the analytes and the IS. Carry over was examined each working day by analyzing blank sample after a calibration spiked standard with concentration 1000 ng/mL.

The recovery was investigated at three levels for the ten benzodiazepines (150, 500, 850 ng/mL) and was calculated by the comparison of extracted spiked sample to unextracted standard solution of the same concentration.

Stability of spiked urine at LOQ concentration, were evaluated in three different pH values (4.8, 6.0, 7.8) at −20 °C for 90 days, at 5 °C for 30 days, at room temperature for 12 h and after three freeze–thaw cycles.

2.7. Matrix effects

For the investigation of signal suppression caused by coeluting matrix substances, ten different blank urine samples spiked with the ten benzodiazepines at two concentration levels (50 and 1000 ng/mL) were analyzed. The absolute and relative responses of the ten benzodiazepines in the spiked urine samples were compared with the results obtained from water samples spiked at the same concentration levels.

2.8. Application in real samples

Ten human urine samples preliminary screened with immunoassay analyzer (Viva-E, Dade Behring) using a cut-off level of 100 ng/mL, were further assayed according to the proposed LC/MS method and finally confirmed with GC/MS.

3. Results and discussion

3.1. Sample preparation

1 mL of each urine sample, was spiked with 50 μL of IS working solution. 500 μL of this sample was diluted with 1 mL ultrapure water. After centrifugation at 2500 rpm for 10 min and filtration with syringe driven filters, 10 μL were injected into the LC/MS system.

3.2. Selectivity – specificity

No endogenous urine components were eluted at the retention time of the analytes, for selectivity. The spiked drugs for the specificity do not interfere with the accurate determination of the ten benzodiazepines in urine.

3.3. Linearity

The calibration curves were linear in the corresponding dynamic ranges with square correlation coefficient (r^2) ≥ 0.981 (Table 2), while the %RSD of the slopes ≤ 8.42. Fig. 1a represents a typical chromatogram of blank urine sample while Fig. 1b represents a typical chromatogram of spiked urine sample at LOQ.

3.4. Limits of detection and quantification

LOD and LOQ levels of all benzodiazepines tested were calculated to range between 10.27–15.61 and 31.13–47.30 ng/mL, respectively, using the equation of the calibration curve (Table 2).

3.5. Precision and accuracy

The %RSD of intra- and interday precision were found to be less than 11.41 and less than 14.88, respectively. The %RSD of intra- and interday accuracy were found to be less than 9.93 and less than 5.72, respectively (Table 3).

3.6. Recovery-stability

The recoveries were found to be higher than 92% for all the benzodiazepines (Table 3).

No significant differences were found between urine samples of three different pH values. The greater difference of the nominal

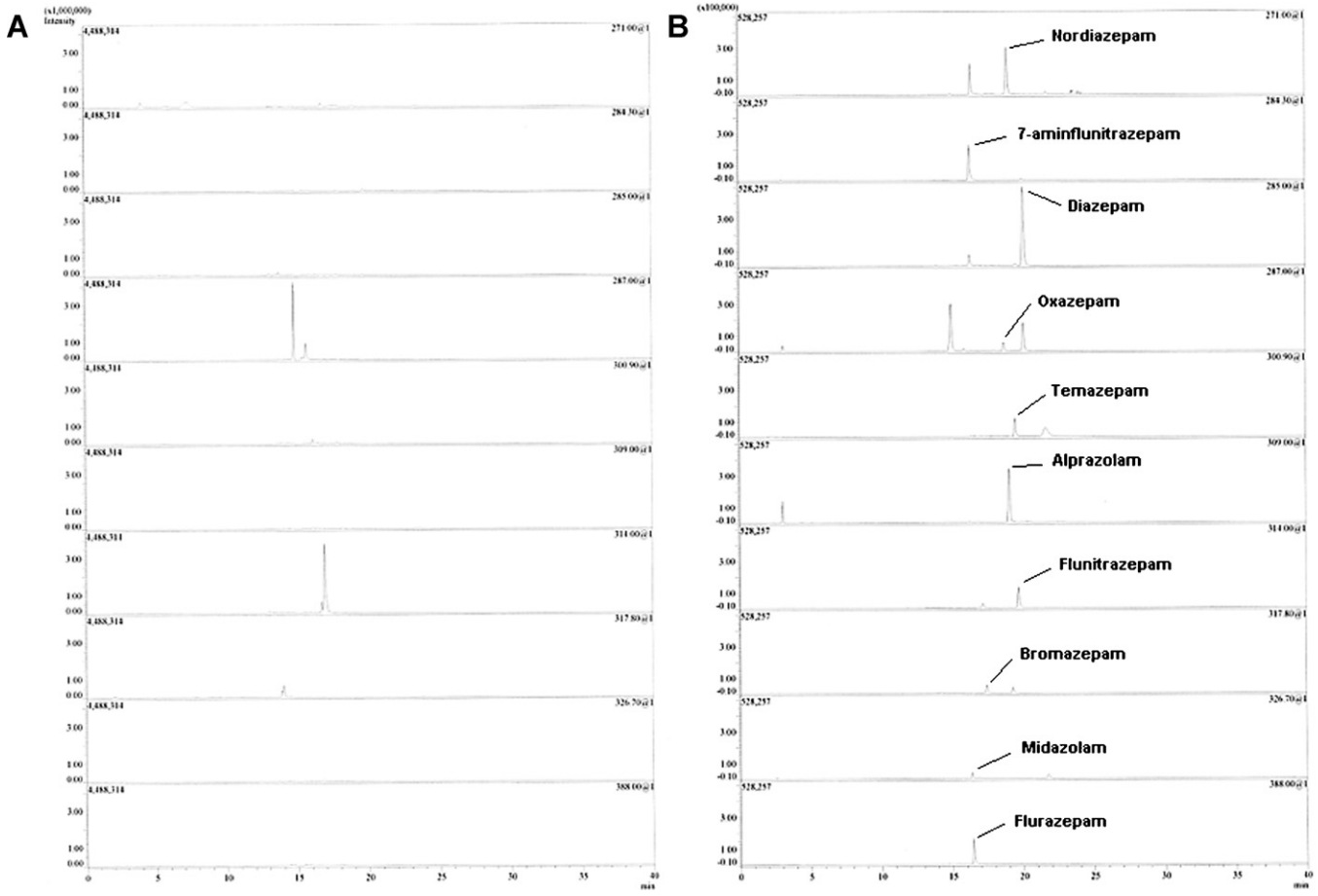

Fig. 1. (A) Representative chromatogram of blank urine, (B) representative chromatogram of spiked urine sample at LOQ (nordiazepam, *m/z* 271, 18.9 min; 7-aminoflunitrazepam, *m/z* 284.3, 16.3 min; diazepam, *m/z* 285, 20.1 min; oxazepam, *m/z* 287, 18.7 min; temazepam, *m/z* 300.9, 19.5 min; alprazolam, *m/z* 309, 19 min; flunitrazepam, *m/z* 314, 19.6 min; bromazepam, *m/z* 317.8, 17.4 min; midazolam, *m/z* 326.7, 16.3 min; and flurazepam, *m/z* 388, 16.4 min).

value for all benzodiazepines in urine after storage of spiked samples at −20 °C for 90 days, at 5 °C for 30 days, at room temperature for 12 h and after three freeze–thaw cycles varied between −6.8% and −0.42%.

3.7. Matrix effect

Urine is an aqueous solution of greater than 95% water, with the remaining constituents, in order of decreasing concentration urea, chloride, Na, K, creatinine and other dissolved ions, inorganic and organic compounds. The small amount of these components which is further diluted, is not expected to affect the column efficacy. An important issue in method development of a quantitative analysis in biological matrix using LC/ESI-MS is the possible occurrence of matrix effects, defined as the effect of coeluting residual matrix components on the ionization of the target analyte. Although the interferences may remain undetected because of the selectivity of the MS detection, they do affect the reproducibility and accuracy of the developed procedure [29,30].

The responses of all benzodiazepines were affected by the matrix no more than 20% while urine components did not appear to affect the column efficacy. The extent of the ion suppression did not depend on the concentration of the analytes. In this case the use of a stable isotope such as diazepam-d5 is equally affected

Table 3
Accuracy, precision and absolute recovery at three quality control levels for the determination of the selected benzodiazepines in human urine.

Benzodiazepine	Concentration levels (ng/mL)	Intraday ($n=6$)		Interday ($n=30$)		% recovery
		Accuracy (%Er)[a]	Precision (%RSD)[b]	Accuracy (%Er)[a]	Precision (%RSD)[b]	
Nordiazepam	150/500/850	1.89/−0.24/0.48	5.42/4.58/2.83	1.87/−0.56/1.20	6.57/3.18/2.57	102/99/101
7-Aminflunitrazepam	150/500/850	3.51/2.75/−5.08	7.48/4.82/0.004	5.72/0.97/−4.44	7.35/5.65/4.26	103/102/95
Diazepam	150/500/850	−1.43/0.31/−1.30	9.57/4.33/1.12	−1.86/2.24/−0.82	6.35/4.52/1.64	98/101/98
Oxazepam	150/500/850	1.11/1.95/−4.18	11.41/3.92/4.78	0.73/3.54/−0.63	7.72/2.76/3.29	101/101/96
Temazepam	150/500/850	−0.94/4.68/−0.46	8.58/7.77/3.62	−4.95/4.76/0.04	14.88/6.26/3.17	99/105/99
Alprazolam	150/500/850	0.96/0.73/−0.36	8.61/4.54/1.78	3.91/0.85/0.98	8.58/4.39/1.91	101/101/99
Flunitrazepam	150/500/850	−1.07/9.93/−3.05	0.78/3.24/−1.25	0.78/3.24/−1.25	6.67/4.84/3.39	92/108/96
Bromazepam	150/500/850	3.35/1.78/−1.08	9.01/5.30/5.65	3.15/2.88/−3.30	10.25/4.14/5.65	103/101/98
Midazolam	150/500/850	1.44/3.63/0.77	9.57/4.82/2.19	−1.31/1.24/0.47	6.85/5.41/2.59	101/103/101
Flurazepam	150/500/850	−0.14/3.36/−1.41	10.01/5.61/2.52	−3.93/2.26/−1.01	13.31/6.58/2.79	99/103/98

[a] %Er, relative percentage error.
[b] %RSD, relative standard deviation.

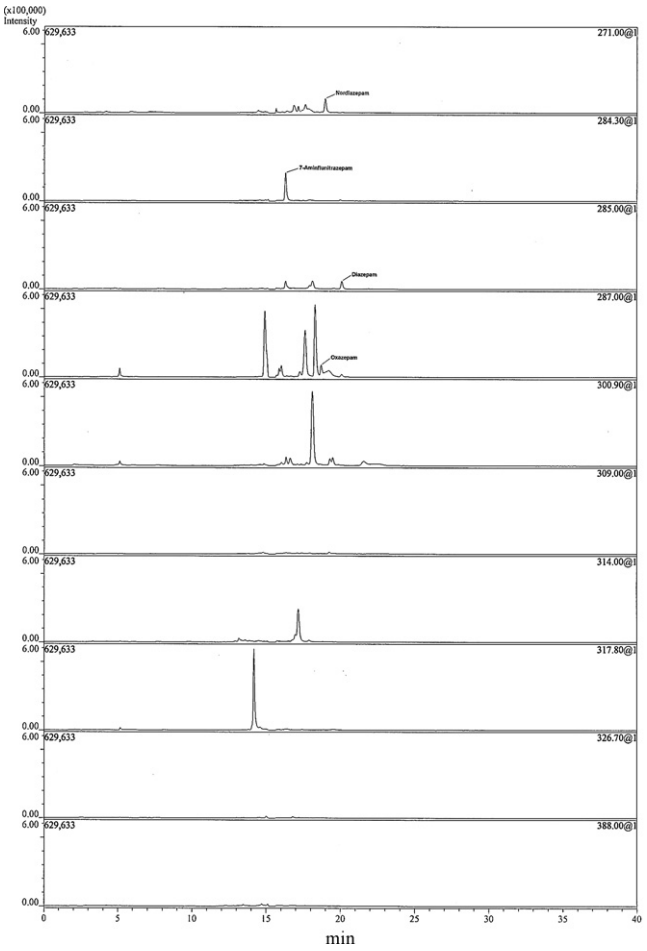

Fig. 2. Representative chromatogram of a case that diazepam nordiazepam, oxazepam, and 7-aminflunitrazepam, were involved (nordiazepam, *m/z* 271, 18.9 min; 7-aminflunitrazepam, *m/z* 284.3, 16.3 min; diazepam, *m/z* 285, 20.1 min; oxazepam, *m/z* 287, 18.7 min).

by fluctuations in detector response and ionization efficiency as the labeled IS and the analytes possess similar physicochemical characteristics.

3.8. Application in real samples

According to the preliminary immunoassay screening, seven samples were found positive and three samples were found negative (<100 ng/mL) for benzodiazepines. Immunoassay was performed using commercially available kit which is based at an Enzyme Multiplied Immunoassay Technique (Emit II Plus). Further assay according to the proposed LC/MS method, revealed that all samples were found to be positive for one or more benzodiazepine. The seven samples found to be positive after immunoassay screening, were determined to be between 120 and 800 ng/mL. In particular, the three samples found negative using the immunoassay method, were found positive for bromazepam, nordiazepam, and bromazepam, respectively using the proposed LC/MS method with concentrations 52.6, 64.7 and 61.2 ng/mL, respectively. All samples were finally confirmed with GC/MS. In this article, a representative case is presented in which diazepam nordiazepam, oxazepam, and 7-aminoflunitrazepam, were involved (Fig. 2).

4. Conclusion

A direct injection LC/ESI-MS method was developed and validated for the identification and quantification of ten benzodiazepines in human urine. The main advantage of the proposed methodology was the lack of an extraction step, as diluted urine samples were directly injected into the LC/MS system. The proposed method is suitable for the replacement of immunoassay screening methods, since it provides higher sensitivity and specificity. The above method was applied successfully to the analysis of ten real samples.

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