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Original Articles

Development of a simultaneous screening method for pregabalin, tramadol, O-desmethyl-tramadol, trihexyphenidyl, oxazepam, midazolam, clonazepam, zolpidem, and buprenorphine in urine using GC-MS

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Abstract:

Confirmation of positive results of immunological urine screening tests for psychotropic drugs is a key element in the fight against drug abuse, drug dependence, and misuse. Our work aims to develop a chromatographic technique for urine analysis by GC-MS for the simultaneous detection and identification of all psychotropic drugs defined by Algerian legislation. The retention times of pregabalin, tramadol, ODMT, trihexyphenidyl, oxazepam, midazolam, clonazepam, zolpidem, and buprenorphine were 12.76, 19.36, 20, 22.81, 23.4, 25.57, 26, 27.22, and 32.19 minutes, respectively. The detection limits estimated by graphical approach were acceptable for confirmation of the CUT-OFF of immunological screening techniques. The present technique offers sufficient sensitivity and efficiency for confirmation of positive results of immunological methods for screening psychotropic drugs in patient urine samples.

Keywords: GC-MS, Urine, Prégabaline, Tramadol, O-Desmethyl-Tramadol, Trihexyphénidyl, Oxazépam, Midazolam, Clonazépam, Zolpidem, Buprénorphine.

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Introduction:

A drug-related trend of medication misuse and diversion is becoming increasingly evident in Algeria. This trend is so pronounced that the trafficking and consumption of psychotropic drugs ranks second after that of cannabis. (ONLCDT, 2022).

The diversity of seizures and diversion of these drugs, first of all, affects the therapeutic classes subject to international control by the International Narcotics Control Board (INCB), including the family of benzodiazepines, certain analgesics such as tramadol and buprenorphine. (INCB, 2022).

However, other therapeutic classes, which are most often used for neurodegenerative diseases, are also diverted, such as certain anti-epileptics (pregabalin), and especially one particular anti-Parkinson's drug, trihexyphenidyl.

The latest statistics provided by the National Office for the Fight against Drugs and Drug Abuse have announced alarming figures: 9,447,154 psychotropic tablets of various brands seized, 31,031 cases dealt with for trafficking, trade, possession and use of psychotropic drugs, during the first ten months of 2022. Psychotropic drugs are at the top of the list of substances circulating in Algeria.

Pregabaline (Lyrica) is the most widely sought-after and consumed product due to its strong euphoric properties when taken at a certain dosage. It is the famous "SAROUKH". (ONLCDT, 2022)

The state decree of 2 Muharram 1443, corresponding to August 11, 2021, was published in Official Journal No. 61, establishing the list of substances and drugs with psychotropic properties with a proven risk of abuse.

A list of substances and drugs with psychotropic properties at a high risk of abuse, addiction, and diversion was published on 08/11/2021. This list includes: buprenorphine, tramadol, the combination of tramadol-paracetamol, clonazepam, pregabalin, trihexyphenidyl, chlordiazepoxide, midazolam, and zolpidem. (Official Journal, 2021).

To this end, the main objective of this work is to develop a urine analysis technique, by gas chromatography coupled to mass spectrometry, targeting the simultaneous search and identification of all drugs with psychotropic properties, as set out in the interministerial decree of 2 Muharram 1443, corresponding to August 11, 2021. As a secondary objective, the application of this technique is to confirm the results rendered positive by immunological screening methods (qualitative) in patients.

To achieve our objectives, we have set the optimal chromatographic conditions and the sample pretreatment that allows us to identify the eight substances cited in a single injection. This work was carried out at the toxicology laboratory of the Central Hospital of the Army Dr. MOHAMED ESSEGHIR NEKKACHE.

Materials:

Reagents and analytical standards:

Reagents:

Methanol (99.8%), acetonitrile (99.9%), 2-propanol (99.8%), 1-chlorobutane (99.8%), trichloromethane (99%), dichloromethane (99.9%), n-hexane (97%), acetone (99.9%), ethyl acetate (99.9%), formic acid (98%), acetic acid (99.7%), hydrochloric acid (37%), sodium sulfate (99%), sodium chloride (99.5%), sodium hydroxide (99.5%), potassium chloride (99.5%), disodium phosphate (99%), boric acid (99.8%), potassium monophosphate (98%) are from the PANREAC® laboratory

Diethyl ether (99.5%) from the HONEYWELL® laboratory

Ammonia (34%) from the CHEMINOVA® laboratory

Ammonium chloride (99.5%) from the NORMAPUR® laboratory

N, O-bis-trimethylsilyl-trifluoroacetamide 99% + trimethylchlorosilane 1% from the SUPELCO® laboratory

Analytical standards:

The analytical standards used for the optimization of the method are:

Tramadol (99.9%) and ODMT (97.1%), powder standards from ANALYTICAL STANDARD SOLUTIONS® laboratory.

Clonazepam (98.5%), Midazolam (98.5%), Oxazepam (98.5%) powder standards from LIPOMED® laboratory.

Buprenorphine (98%) UNODC standard in powder form.

Pregabalin (100%) and Trihexyphenidyl (100%) are raw powders manufactured by PHARMALLIANCE® laboratory

Zolpidem (100%) raw powder manufactured by GENRICLAB® laboratory.

The internal standard is N-acetyl-paroxetine at a concentration of 200 mg/l prepared in the toxicology laboratory of the HCA from an acetylation of paroxetine which is supplied by A2S® laboratory.

Biological material:

The biological material used for the development of our method is urine free of the psychotropic drugs being sought. It is the urine of subjects whose immunological research result has been negative.

It is used to prepare the mixtures of analytical standards used during the optimization of the technique.

Urine samples from patients are taken at the laboratory. Each subject will have a new plastic container for collecting urine. The samples are stored at 2-8°C if the analysis is scheduled within 3 days of collection If the analysis is delayed, the storage will be carried out at -20°C.

Equipment:

- Gas chromatograph (GC) from the SHIMADZU® NEXIS GC-2030 brand, coupled to a quadrupole mass spectrometer (QMS) from the SHIMADZU® NEXIS GCMS-QP2030 NX brand, used for the optimization of the analysis method.

- Column: RESTEK® RXI-5SIL MS, 30 meters * 0.25 mm id, 0.25 um df.
- Software: GC-MS REAL TIME ANALYSIS.
- Precision balance (SARTORIUS®).
- Aspirator hood: ERLAB®, CAPTAIR SMART 321.
- Rotator: SNIJDERS SCIENTIFIC® 34528.
- Centrifuge: EPPENDORF® centrifuge 5702 RH.
- Rota vape: EPPENDORF® concentrateur plus.
- pH meter: METTLER TOLEDO® SEVEN COMPACT.
- Electronic laboratory hot block: STUART EQUIPMENT® SBH200D/3.
- Vortex: BIO-RAD® BR-2000.

Methods:

Identification criteria for molecules:

In order to identify the targeted molecules, we relied on the following criteria:

The search for specific fragments after derivatization of the molecule by BSTFA 99% + TMCS 1%, which are obtained by fragmentation of the molecular ions, in the ionization source with an ionization energy of 70 electron volts (eV) in the case of this method.

The mass spectrum by comparing the sample spectra with the libraries available in the GC-MS database (Forensic toxicology 1_e1, Forensic toxicology_v2, Benzodiazépine1_nci, W11n17main1 and W11n17main2)

Preparation of stock and working solutions:

Stock solutions at 1 mg/mL were prepared in methanol from the analytical standards.

Subsequently, working solutions were prepared from the stock solutions by dissolving them in urine free of the psychotropic drugs being sought:

- 25 µL of the stock solution qsp 5 mL urine for molecules at 5 µg/mL.
- 5 µL of the stock solution qsp 5 mL urine for molecules at 1 µL/mL.

Concentration of working solutions in analytical standards:

Clonazepam: 5 µg/mL.

Pregabalin: 5 µg/mL.

Buprenorphine: 1 µg/mL.

Tramadol: 1 µg/mL.

ODMT: 1 µg/mL.

Midazolam: 1 µg/mL.

Oxazepam: 1 µg/mL.

Zolpidem: 1 µg/mL.

Trihexyphenidyl: 1 µg/mL.

Sample treatment protocol:

Liquid-liquid extraction:

Two types of sample treatment were optimized for the extraction of abuse drugs from a urine sample:

In a 10 mL screw-top tube, place in order:

- 1 mL of urine sample (or control point) + 8 mL of extraction solvent (dichloromethane/isopropanol, 8/2, v/v);
- Shake 3 times by inversion;
- Extraction by rotator in vertical position for 10 minutes;
- Centrifuge at 3000 rpm for 3 minutes and then remove the upper phase (urine);
- Transfer the lower organic phase to a 10 mL conical glass tube and dry at 40°C under a gentle stream of nitrogen.

Derivatization of the evaporation residue:

Add 60 µL of acetonitrile + 40 µL (BSTFA 99%+TMCS 1%) to the evaporation residue, shake and place in an oven at 70°C for 30 minutes

Cool and inject 1 µl into the GC-MS system.

Chromatographic conditions:

- Injector temperature: 250°C splitless.
- Oven program: 60°C (2 minutes) to 10°C/minute ramp to 320°C (35 minutes).
- Transfer line temperature: 240°C.
- Detector temperature: 200°C.
- Gas (helium): 1.34 mL/minute.
- Injected volume: 1 µL.
- Start time: 3 minutes
- Analysis time: 35 minutes.

Estimation of the detection and quantification limits:

The estimation of the detection and quantification limits of our technique is based on the graphical approach of the SFSTP'1992 validation guide for analytical methods (Caporal-Gautier et al., 1992).

The detection and quantification limits are estimated from the background noise of the

graphical recording on a sample, according to the following equations:

LOD = 3 * hmax * R (associated risk remains below 0.13 %), and

LOQ = 10 * hmax * R (associated risk remains below 0.5 %),

With hmax **(figure 01)** the maximum background noise height over a window corresponding to 10 half-height peak widths on either side of the Tr.

R the quantity/signal response factor, expressed in mass quantity/height.

These estimates can be validated by injecting concentrations close to the calculated limits

Liquid-liquid extraction (LLE) trials on patients :

To test our optimized LLE technique, we applied it to four patients, for confirmation of the positive results for tramadol and pregabalin obtained by immunological screening methods (lateral flow immunochromatography, EMIT).

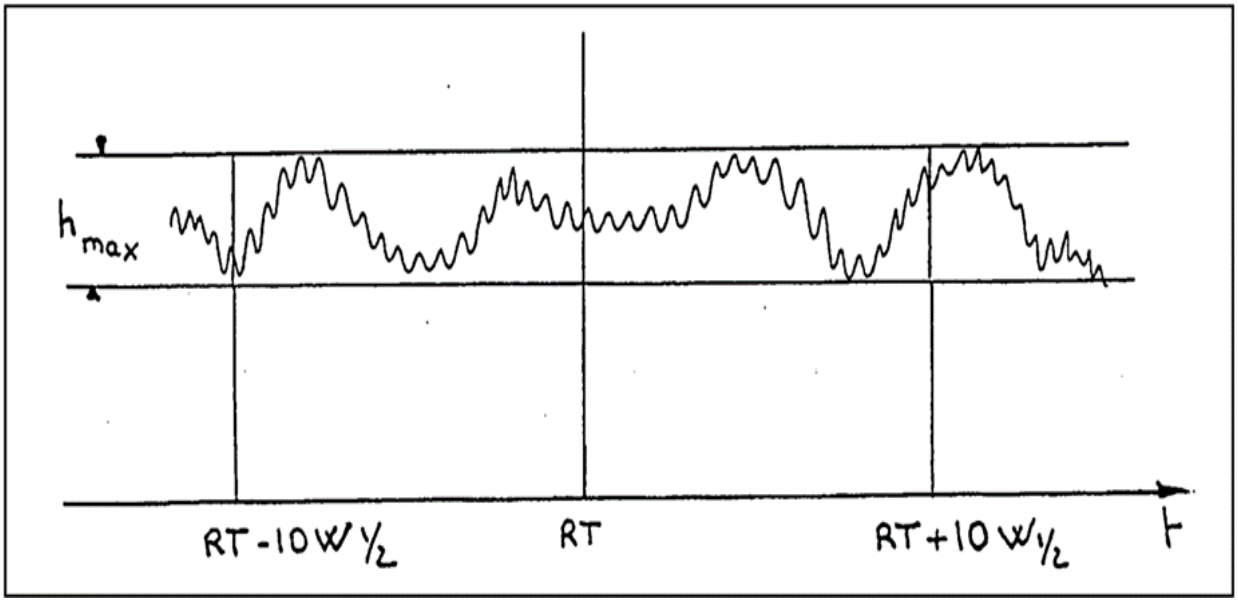

Figure 1. Estimation of detection limits using the graphical approach after extraction of the urine sample using the optimized ELL technique.

Results:

Results of the optimization of liquid-liquid extraction (LLE):

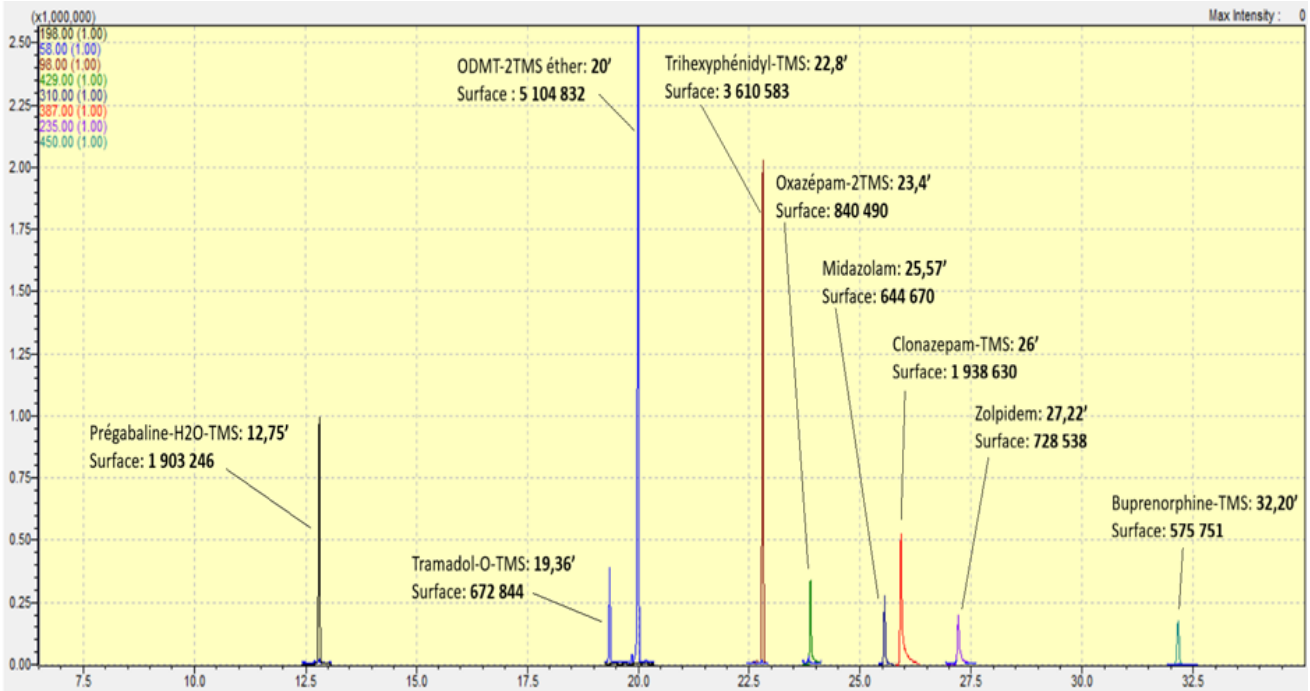

Figure 2. Chromatogram of molecules by major fragments in SCAN mode, with areas and retention times, after extraction by the mixture (dichloromethane/isopropanol, 8/2, V/V).

Figure 2 shows that the use of the solvent mixture (dichloromethane/isopropanol, 8/2, v/v) for sample treatment gave good results. We were able to obtain fine Gaussian-shaped peaks with acceptable signals, which allows us to reach the desired detection and quantification limits.

It is also noted that the pregabalin, tramadol, ODMT, oxazepam, clonazepam, and buprenorphine were derivatized, while midazolam and zolpidem remained in their free, non-derivatized forms.

Table 1. Determination of the half-height peak width (w1/2) and the maximum background noise height (hmax) for the estimation of detection and quantification limits using the SFSTP 1992 graphical approach.

	Concentration (ng/mL)	Signal (nm)	R	H_{max} (nm)	LOD (ng/mL)	Cutoff (ng/mL)
Prégabaline	10 000	850 924	0,01175	11 977	**420**	**500**
Tramadol	1000	766 924	0,0013	52 719	**210**	**100**
ODMT	1000	5 383 748	$1,85 * 10^{-4}$	35 487	**19,77**	**600**
Trihexyphénidyl	1000	3 999 639	$25 * 10^{-5}$	7 278	**5,45**	/
Oxazépam	1000	546 332	$18,3 * 10^{-4}$	5 847	**32,11**	**350**
Midazolam	1000	545 487	$18,33 *10^{-4}$	10 541	**57,97**	**160**
Zolpidem	1000	485 547	$2 * 10^{-3}$	5 286	**32,66**	**50**
Clonazépam	10 000	1 540 693	$65 * 10^{-4}$	45 521	**886,37**	**1100**
Buprénorphine	1000	359 473	$27,8*10^{-3}$	3 801	**31,72**	**20**

Estimation of the detection and quantification limits:

Table 1 shows the detection and quantification limits estimated by graphical approach according to the SFSTP'1992 guide for validation of analytical methods, after LLE of the molecules.

Application of the optimized LLE technique to patient samples:

The LLE trial on the urine samples of the two patients whose immunological screening results were positive gave very good results. The technique confirmed the use of tramadol and/or pregabalin in the patients.

The technique was reliable for high concentrations of pregabalin and tramadol in patient 1:

Surface of the major 198 fragment of pregabalin: 7,623,441

Surface of the major 58 fragment of tramadol: 47,409,015 (with detector saturation)

Surface of the major 58 fragment of ODMT: 4,392,106

Even for low concentrations of pregabalin and tramadol, LLE gave reliable results:

Surface of the major 198 fragment of pregabalin: 176,173

Surface of the major 58 fragment of tramadol: 299,101

To avoid the problem of detector saturation with high concentrations of tramadol, we propose to dilute samples that are highly concentrated in tramadol (the EMIT technique used for screening gives semi-quantitative results that allow an estimate of the concentration of the sample).

Among the strengths of our study, we can cite:

The development of an analytical technique by GC-MS of eight molecules, despite the absence of scientific articles that group the simultaneous analysis of these molecules.

The use of SPE extraction allowed us to increase the sensitivity for tramadol and buprenorphine compared to liquid-liquid extraction, and also allowed us to extract pregabalin in the aqueous phase recovered in the rinsing solution (hydrosoluble properties).

Chromatographic conditions allowing for simultaneous analysis in a single injection and in a relatively short time of 35 minutes.

Discussion:

Optimization of chromatographic conditions:

The analysis of the molecules with the chromatographic conditions of the Grapp et al 2016 technique gave poor results for most of the molecules, with poorly shaped chromatographic peaks and very weak signals for: clonazepam, pregabalin, trihexyphenidyl, oxazepam, midazolam, and zolpidem, which does not allow the desired concentrations to be reached after extraction.

The application of the chromatographic conditions proposed by SHIMADZU for forensic analysis by GC-MS, improved the signal and shape of the chromatographic peaks of all molecules with a change in retention times, except for clonazepam, whose peak remains wide.

The use of ethyl acetate as a solvent for recovering the evaporation residue, did not allow the derivatization of: clonazepam, pregabalin, and trihexyphenidyl, which led us to substitute it with acetonitrile, which is more polar and allowed their derivatization

Optimization of the simultaneous extraction of molecules:

In order to extract the highly water-soluble molecules (pregabalin, tramadol, ODMT, and midazolam) within this heterogeneous family by organic solvents, we tried several liquid-liquid extractions with mixtures of solvents of different polarities (polar and apolar).

The extraction of the urine sample by the Alahyari technique using a mixture (chloroform / acetone, V/V) certainly improved the results for ODMT, Trihexyphenidyl, Oxazepam, Midazolam and Zolpidem, but with absence of the peaks corresponding to pregabalin, tramadol, clonazepam and buprenorphine.

By the process of Persona et al., 2015 which uses the solvent mixture (chlorobutane / isopropanol, 9/1, V/V), we were able to obtain chromatographic peaks corresponding to pregabalin, tramadol and oxazepam.

However, we still note the absence of the signal corresponding to clonazepam, which seems to be due to its insolubility in the solvents used.

The use of a polar solvent (isopropanol) allowed the extraction of pregabalin and tramadol (highly water-soluble molecules).

With the second mixture proposed by Persona et al 2015 (Diethyl ether/isopropanol, 8/2, V/V), we were able to extract all our molecules with improved signal for pregabalin and a clear decrease in the signals for tramadol, ODMT, trihexyphenidyl, oxazepam, midazolam, zolpidem and buprenorphine.

We also noted the appearance of the chromatographic peak corresponding to clonazepam with a very poor appearance (wide and distorted peak).

The use of the mixture of dichloromethan/isopropanol showed that dichloromethan actually gives better results compared to chloroform, acetone and ether.

With this mixture, we were able to improve the signal for pregabalin, tramadol, ODMT while keeping acceptable signals for the other molecules.

After testing several buffers at different pH values, we finally opted for the use of Na2HPO4 as a buffer in the extraction. Na2HPO4 is characterized by its strong buffering capacity and a neutral pH that allows the extraction of all benzodiazepines.

The use of Na2HPO4 as a buffer significantly improved the signals and appearances of all the molecules.

Optimization of the derivatization:

The use of 50 µL (BSTFA 99%+TMCS 1%) + 50 µL ACN for the recovery and derivatization of the evaporation residue gave the best results for pregabalin, tramadol, ODMT and trihexyphenidyl, but not for clonazepam and oxazepam, for which the signals were very weak. This led us to opt for a derivatization with 40 µL (BSTFA 99%+TMCS 1%) + 60 µL ACN, which gives a better signal for oxazepam and zolpidem, and signals close to the best signals for the other molecules.

The signals of pregabalin, tramadol, ODMT, trihexyphenidyl, oxazepam, zolpidem and buprenorphine evolved proportionally with the

increase in the derivatization temperature up to 80°C (optimal derivatization temperature).

At a derivatization temperature of 90°C, a loss of signal was observed for pregabalin and a clear decrease for the other molecules mentioned above.

For clonazepam, the optimal derivatization temperature is 70°C, after which the signal decreases inversely with increasing temperature.

We finally opted for a derivatization temperature of 70°C for all molecules, as this temperature provides signals for pregabalin, tramadol, ODMT, trihexyphenidyl, oxazepam, zolpidem and buprenorphine that are close to those of 80°C and allows for a better signal for clonazepam, with which we found a lot of difficulty during the optimization of the technique.

Application of the optimized ELL technique to patient samples:

The ELL assay on the urine samples of the two patients whose immunological screening results were positive, gave very good results, the technique allowed to confirm the intake of tramadol and pregabalin in both patients.

The technique was reliable for the high concentrations of pregabalin and tramadol in patient 1:

- Surface of the major fragment 198 of pregabalin: 7,623,441;
- Surface of the major fragment 58 of tramadol: 47,409,015 (with detector saturation);
- Surface of the major fragment 58 of ODMT: 4,392,106.
- Even for the low concentrations (patient 2) of pregabalin and tramadol, ELL gave reliable results:
- Surface of the major fragment 198 of pregabalin: 176,173

To avoid the problem of detector saturation with high concentrations of tramadol, it is proposed to dilute the samples that are highly concentrated in tramadol (the EMIT technique used for screening provides semi-quantitative results that allow for an estimate of the concentration of the sample).

Among the strengths of our study, we can cite:

- The development of a GC-MS analysis technique for eight molecules, despite the absence of scientific articles that combine the simultaneous analysis of these molecules.

- Chromatographic conditions that allow for simultaneous analysis in a single injection and in a relatively short time of 35 minutes.

- Surface of the major fragment 58 of tramadol: 299,101

As for the weaknesses:

- The sensitivity of the technique could be improved for low concentrations of tramadol.

- The technique requires expensive equipment.

It is possible to expose the weaknesses regarding the validation of the method as well as the population size, which are due to the unavailability of the equipment (GC-MS).

Conclusion:

The objective of this work was to develop a GC-MS analysis technique for urine that would allow for the simultaneous detection and identification of all psychoactive drugs listed in the state decree of 2 Muharram 1443, corresponding to August 11, 2021.

The ELL technique has a high enough sensitivity for the eight target substances to allow for confirmation of positive results from immunological screening techniques.

A separation of the target analytes was performed with chromatographic conditions allowing an analysis in a relatively short time (35 minutes).

We optimized a single derivatization protocol using a mixture of 60 µL (BSTFA 99% + TMCS 1%) + 40 µL ACN in 30 minutes with an optimal temperature of 70°C, allowing identification of all target analytes with the best possible sensitivity and specificity.

The optimized method has sufficient detection limits for the confirmation of positive results from immunological screening techniques.

The application of this optimized method to two urine samples from patients confirmed the presence of tramadol and pregabalin, which were positive for immunological screening methods.

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