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Rapid and highly sensitive determination of clonazepam and 7-aminoclonazepam in whole blood using gas chromatography with negative-ion chemical ionization mass spectrometry

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Gas chromatography combined with negative-ion chemical ionization mass spectrometry (GC/NICI-MS) improves the sensitivity and specificity for quantitation of clonazepam and its metabolite 7-aminoclonazepam in whole blood, significantly exceeding these parameters achieved by traditional electron impact ionization mass spectrometric detection. Common liquid-liquid extraction and derivatization parameters such as nature of the extraction solvent, sample pH, extraction time, derivatization time and temperature were optimized. Analytes were extracted using n-butyl acetate, then derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) followed by GC/NICI-MS analysis. The limits of detection of clonazepam and 7-aminoclonazepam in blood sample were 1.0 and 1.4 ng mL^{-1}, respectively, and the calibration curves were linear in the concentration range 5–200 ng mL^{-1} with $r^2 > 0.995$. The relative standard deviations during investigation of samples used for quality control were lower than 7.0% (at n = 20). Obtained results showed that the developed GC/NICI-MS method is relatively simple, accurate, sensitive and selective. Finally, it was demonstrated that this method is applicable for the determination of trace concentrations of clonazepam and 7-aminoclonazepam in whole blood samples.

Key words: clonazepam, 7-aminoclonazepam, blood, LLE, GC/NICI-MS

INTRODUCTION

Clonazepam and its metabolite 7-aminoclonazepam are benzodiazepine, which exhibits many of the characteristic pharmacological properties that are common for this class of compounds. Clonazepam has been shown to decrease seizure activity, to reduce anxiety, to induce muscle weakness, and even induce sleep or hypnosis. Because of these effects, clonazepam has been identified as a potential drug [1–3]. Moreover, physicians are also using clonazepam in the treatment of anxiety, mania, panic disorders, and schizophrenia because of its sedative and anxiolytic properties [4].

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Several chromatographic methods for the analysis of clonazepam and its metabolite in biological samples have been reported including high-performance liquid chromatography (HPLC) combined with conventional spectrophotometric [5, 6], single (LC-MS) or tandem mass spectrometric (LC-MS/MS) detection [7–9], gas chromatography using electron capture or electron impact MS (GC/EI-MS) detection [10, 11]. Gas chromatography coupled with negative-ion chemical ionization MS (GC/NICI-MS) offers much better sensitivity than the conventional GC/EI-MS technique and it has been successfully applied for the detection of different analytes in blood samples [12, 13]. Negative ion chemical ionization MS can improve the sensitivity by a factor of several thousand if compared with positive ion chemical ionization MS or electron capture detection, especially for the determination of compounds with electronegative moieties, such as halogen atoms in the xenobiotic itself, or after corresponding derivatization [14].

Direct determination of clonazepam and 7-aminoclonazepam in biological samples is complicated due to significant matrix interferences and relatively low concentrations of the analytes. However, there are a limited number of sample preparation procedures, which are suitable for effective isolation / preconcentration of clonazepam and its metabolite from biological samples. The most widely used sample preparation techniques are solid-phase extraction (SPE) [9, 15] and liquid-liquid extraction (LLE) [16]. LLE offers some advantages including low consumption of sample, high speed, simple device, easy operation, and low costs.

By GC determination of clonazepam and 7-aminoclonazepam in biological samples derivatization step is often preferable because it enables better chromatographic resolution and higher detection sensitivity [16, 17]. A method based on the derivatization with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) followed by GC-MS analysis was developed for the analysis of common analytes. The MTBSTFA forms sensitively detected, reproducible and stable derivatives, regardless of whether the analytes molecule has active hydrogen atoms in the secondary amine group and / or the hydroxyl group [18]. This is very important for the evaluation of final results of the whole blood in forensic toxicology and / or clinical medicine, but many research papers present only the evaluation of main clonazepam. Moreover, there are no reports about the application of MTBSTFA as a derivatization reagent for the analysis of 7-aminoclonazepam by GC/NICI-MS methods.

Thus, because of the reasons mentioned above, the aim of this study was to develop a simple procedure for rapid and sensitive determination of clonazepam and 7-aminoclonazepam in the whole blood. The proposed procedure is based on LLE with n-butyl acetate, subsequent derivatization of the extracted analytes with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide followed by GC/NICI-MS analysis and evaluation of stability of samples.

MATERIALS AND METHODS

Reagents

Clonazepam and 7-aminoclonazepam were obtained from Lipomed-Services to Medicine (CH-4144 Arlesheim, Switzerland), an ampoule of 7-aminoclonazepam-d4 (100 μg mL^{-1}) was purchased from Cerilliant Corporation (Round Rock, USA). Silylating reagent N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) of analytical grade was purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Methanol (MeOH), n-butyl acetate, ethyl acetate, chloroform, dichloromethane, isooctane, toluene, phosphoric acid (H_3PO_4), potassium hydroxide (KOH) and potassium hydrogen phosphate (K_2HPO_4) of analytical grade were supplied by Merck (Darmstadt, Germany). In the study, fresh human whole blood from the National Blood Center of Lithuania and stored (drug-free) human whole blood from authentic samples submitted to the author's laboratory were used.

Preparation of standard stock solutions

Clonazepam and 7-aminoclonazepam standard were accurately weighed, transferred to volumetric flasks and dissolved in MeOH to make individual stock solutions of 1.0 mg mL^{-1}. These solutions were thoroughly mixed and stored at –20 °C in tightly closed bottles until their use. Working solutions of analyte at the concentration of 5.0, 10.0, 20.0, 50.0, 70.0, 100.0, 120.0, 150.0 and 200.0 ng mL^{-1} were diluted from the stock solution with MeOH and used for spiking whole blood.

Preparation of spiked whole blood samples

The standard working solutions of 1.0 mL volume were taken into a 5 mL glass tube and evaporated to dryness under a gentle stream of nitrogen. Then the residues were reconstituted with 1.0 mL of drug-free human whole blood, which was thawed to room temperature in advance, to give final analyte concentrations of 5.0, 10.0, 20.0, 50.0, 70.0, 100.0, 120.0, 150.0 and 200.0 ng mL^{-1}. This sequence of spiked human whole blood solutions was considered as the matrix-matched calibration standard and four concentrations of 10.0, 50.0, 100.0 and 150.0 ng mL^{-1} of analyte in whole blood were considered as quality control (QC) samples.

Preparation of internal standard solution

Internal standard (IS) stock solution was prepared by adding 7-aminoclonazepam-d4 (100 μg mL^{-1}) deuterated analogue in a volumetric flask and diluted in MeOH, the final concentration of IS per sample was 50 ng mL^{-1}. IS stock solution was stored in dark at –20 °C.

Instrumentation and chromatographic conditions

Identification of clonazepam and its metabolite by GC/NICI-MS was carried out using an Agilent Technologies-7890A (Folsom, CA, USA) gas chromatograph coupled to a detector 5975C EI-MS and another detector 5975C NCI-MS. Injection was performed manually using combined

split / splitless mode and injector temperature of 250 °C. The chromatographic separation was performed using a DB-5-HT capillary column (30 m × 0.320 mm I. D., 0.10 μm film thickness). The initial temperature of the analytical column was 180 °C, which was then increased by 40 °C per min to 325 °C and hold stable heating for one min. The chromatographic runtime was 3.9 min. The carrier helium gas (purity 99.9996%) was flowed at a constant flow-rate of 3.5 mL min^{-1} after pulsed flow injection in a splitless mode (1.0 μL).

NICI-MS modes mass spectrometric conditions

The NICI mode was used with methane 99.9995 % as a reagent gas in all MS measurements. The flow controller of methane was set to 40% of the maximal Agilent default value in NICI mode and 20% in positive-ion chemical ionization (PICI). Pretuning of the MS system in PICI mode was performed prior to each analysis to identify possible air leaks and moisture. In NICI-MS multiplier voltage was 1 600 V, emission 45 μA, electron energy 148 eV, repeller 2.7 V and ion focus 130 V. MS detector transfer line temperature was 300 °C, MS quadrupole and MS source temperatures were 150 °C. All quantitative analyses were performed in the selected ion monitoring (SIM) mode. In one separate SIM ion-time-window was used. In all scanning (SCAN) mode experiments a mass range of 30–700 amu was covered.

Optimization of sample pretreatment

100 μL of 0.5 M K_2HPO_4 solution (pH 9.5, sample pH was optimized as it is presented in Results and Discussion) and 300 μL of n-butyl acetate were added to 200 μL of whole blood and 10 μL of IS. The contents of the test tubes were rapidly and simultaneously vortex-mixed at speed of 2 000 rad min^{-1} for 20 seconds (extraction time was optimized as it is presented in Results and Discussion) in a multitube vortexer without removing the test tubes from the rack. After centrifugation (3 500 g, 5 min), 100 μL of the supernatant and 10 μL of MTBSTFA were added to 2.0 mL autosampler vials containing 200 μL inserts. The samples were rapidly vortex-mixed and the capped vials were kept in a heating block for 40 min at 90 °C (derivatization time and temperature were optimized as it is presented in Results and Discussion). One microliter of preparation samples was injected into the GC/NICI-MS system.

Linearity

Quantitation of clonazepam and 7-aminoclonazepam was performed by the internal standard method. The calibration curves were fitted by linear least square regression of the responses. The peak area ratio of analyte to IS (7-aminoclonazepam-d4) was considered as the response. Peak area ratios were determined for the control blood preparations.

Recovery and precision

The extraction recovery, precision and relative standard deviation (RSD) data at four different concentration levels were calculated for blood samples. The QC levels were determined by comparing the peak area and height ratio of the analytes to the IS in samples. Drug-free human whole blood samples were spiked with the analytes prior to extraction. To investigate the repeatability of the method, six replicates of spiked QC human whole blood samples with different concentrations were extracted and analyzed within one day. Single-day precision (at n = 6) and time-different intermediate precision (at n = 20) were evaluated by performing replicates of spiked QC samples within the period of twenty days.

Stability

The stability of standard solutions, QC samples, and human blood extracts was evaluated at room temperature, after 24 h in an autosampler. In addition, the stability of samples after thirty, sixty and ninety days of storage in a –20 °C freezer was tested. The QC samples of the medium-level concentration (100 ng mL^{-1}) were used in this study.

RESULTS AND DISCUSSION

For the optimization of GC separation, NICI-MS detection, LLE and derivatization conditions, whole blood samples spiked with individual analytes at 50 ng mL^{-1} concentration level were used. The main LLE parameters that were optimized include nature of extraction solvent, sample pH and extraction time. Several parameters of the derivatization procedure were also optimized. Finally, linearity, recovery tests, precision, and stability of QC samples were evaluated.

Instrumental parameters

Optimisation of GC oven program

The optimum initial column oven temperature was found to be 180 °C. Starting with higher initial temperature (e. g. 250 °C) and higher ramp resulted in early elution of the analyte, but signal-to-noise and peaks purity were affected. The 7-aminoclonazepam and clonazepam peaks were also affected by coeluted solvent and a single peak shape. Similarly, with too low initial temperature (e. g. 140 °C) the broadening of peaks for 7-aminoclonazepam and clonazepam was observed. Width of peaks was higher by 3% and 5% and height of peaks was lower by 8% and 10% if compared with optimized conditions for 7-aminoclonazepam and clonazepam, respectively. Fast ramping at the rate of 40 °C min^{-1} to 325 °C with a hold for 1 min could remove entire solvent traces from the column before elution of 7-aminoclonazepam and clonazepam. Under the optimized GC conditions, the derivatives of both analytes were completely separated within 3.9 min as shown in Fig. 1. As can be seen, each compound displayed a single peak with a good peak shape.

Optimisation of chromatographic and MS parameters

Robustness of the entire method was studied by changing several chromatographic and MS parameters: flow rate of carrier gas from 0.5 to 7.0 mL min^{-1}, injector temperature from 220 to

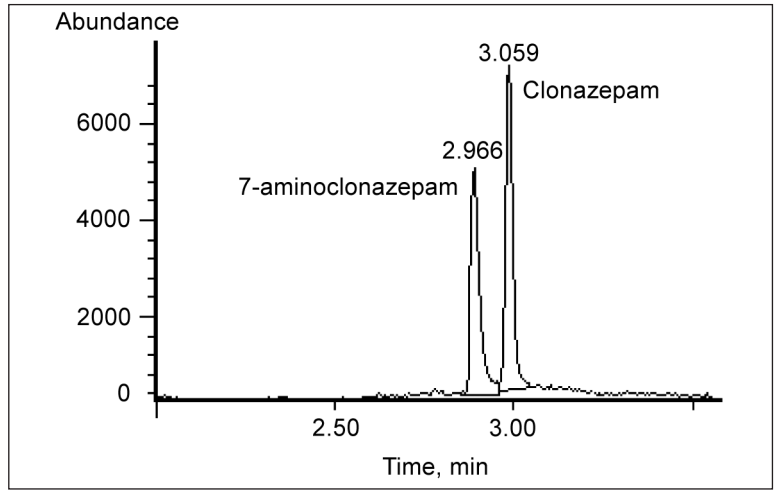

Fig. 1. Chromatogram of whole blood sample spiked with 50 ng mL^{-1} concentration of each analyte

300 °C, and 20% higher detector voltage to 1 920 V. The differences of the mean areas of the analyte, for each parameters of the method changed, as well as their standard deviations were calculated. A single parameter or a combination changed all chromatographic and MS results. Under the optimised conditions for 7-aminoclonazepam and clonazepam, the flow rate of carrier gas was 3.5 mL min^{-1}, injector temperature 250 °C and NICI-MS multiplier voltage was 1 600 V or 5% higher.

The interpretation of NICI mass spectra

The mass spectra of the MTBSTFA derivatives of clonazepam and 7-aminoclonazepam are shown in Fig. 2.

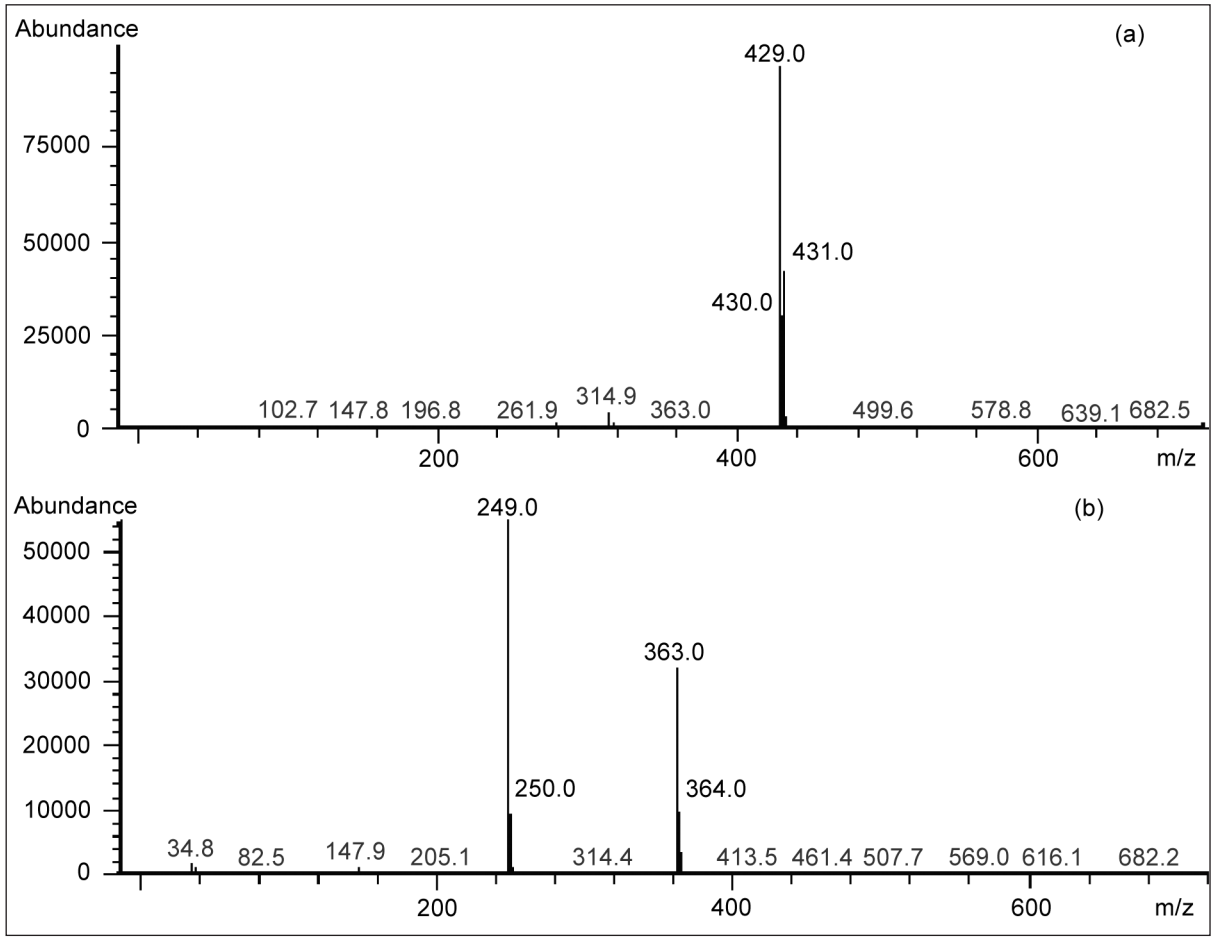

Fig. 2. Mass spectra of the MTBSTFA derivatives of clonazepam (a) and 7-aminoclonazepam (b)

Table 1. **SIM parameters of GC/NICI-MS run**

Analyte	Dwell time, ms	Cycle time[a], cycles per sec	SIM ions, m/z
Clonazepam	40	2.88	429; 431; 430
7-aminoclonazepam	40	2.88	249; 363; 364
7-aminoclonazepam-d4	40	2.88	253; 367

[a] Cycle time is expressed as cycles per second at a time interval corresponding to SIM ion-time-window.

Clonazepam derivative in the NICI-MS mode gave the main $[M-H]^-$, $[M]^-$ and $[M+H]^-$ ions. The full fragmentation is illustrated in Fig. 2(a). The methane induced NICI mass spectrum shows the quasimolecular ions $[M-H]^-$ (m/z 429) and $[M+H]^-$ (m/z 431) with the base peak intensity of 100.0% and 41.2 ± 1.6% (SD_n = 10), respectively. The molecular m/z 430 ion with 30.1 ± 1.4% (SD_n = 10) intensity was also observed. Virtually, it is the only confirmation factor for clonazepam, resulting from reaction in NICI mode.

The ions with m/z 249, 250, 363, 364 and intensities of 100.0%, 10.5 ± 1.9%, 38.2 ± 4.4%, and 11.9 ± 1.3% (SD_n = 10), respectively, are the typical results of fragmentation of 7-aminoclonazepam, without the molecular ion. Full fragmentation is illustrated in Fig. 2(b). The base peak of m/z 249 is assumed to be an ion of $[M-1TBDMS]^-$ derived from the fragmented molecular ion at m/z 363, formed by loss of one TBDMS (tert-butyl-dimethylsilyl) group. The quasimolecular ion at m/z 250 was obtained from m/z 364 ion, and this is a typical fragmentation of 7-aminoclonazepam. The fragmental pattern of 7-aminoclonazepam-d4 was similar to that of 7-aminoclonazepam compound.

Three and two ions were chosen for GC/NICI-MS analysis and the highest abundant mass ion was used for quantitative analysis. Complete SIM parameters, target and qualifier ions of the compounds are shown in Table 1.

Selection of organic solvent for extraction

The most important task in the optimization of LLE procedure is the selection of suitable extraction solvent. The main solvent properties that need to be considered are: (i) low vapor pressure in order to avoid evaporation during extraction, (ii) high solubility of analytes, and (iii) compatibility with direct injection into GC/NICI-MS. Several water-immiscible (blood samples) organic solvents commonly used in the traditional LLE, namely n-butyl acetate, ethyl acetate, chloroform, dichloromethane, isooctane and toluene, were investigated in this work. Each solvent was examined at room temperature using spiked blood samples at a concentration of 50 ng mL^{-1} of each analyte. The obtained results are shown in Fig. 3. Among all extraction solvents studied, n-butyl acetate has demonstrated the best extraction efficiency for both analytes. In addition, all solvents have shown similar retention times with the analytes except n-butyl acetate. Thus, n-butyl acetate was selected as the extraction solvent. Besides, the use of n-butyl acetate as the extraction solvent provides other advantages, such as excellent extraction efficiency and a sharp chromatography peak.

Optimization of sample pH

Since both analytes are weakly basic (pK_a values for clonazepam are 1.5 and 10.5 [15], for 7-aminoclonazepam, however, they were not available), pH of the sample solution should be a very important parameter in LLE. The effect of sample pH on the extraction efficiency was investigated in phosphate buffer solutions prepared by neutralization of 0.5 M K_2HPO_4 with 4 M H_3PO_4 (for pH range of 1.0–9.0) or with 4 M KOH (for pH range of 10.0–11.0). The results are shown in Fig. 4. As expected, the peak areas of both analytes

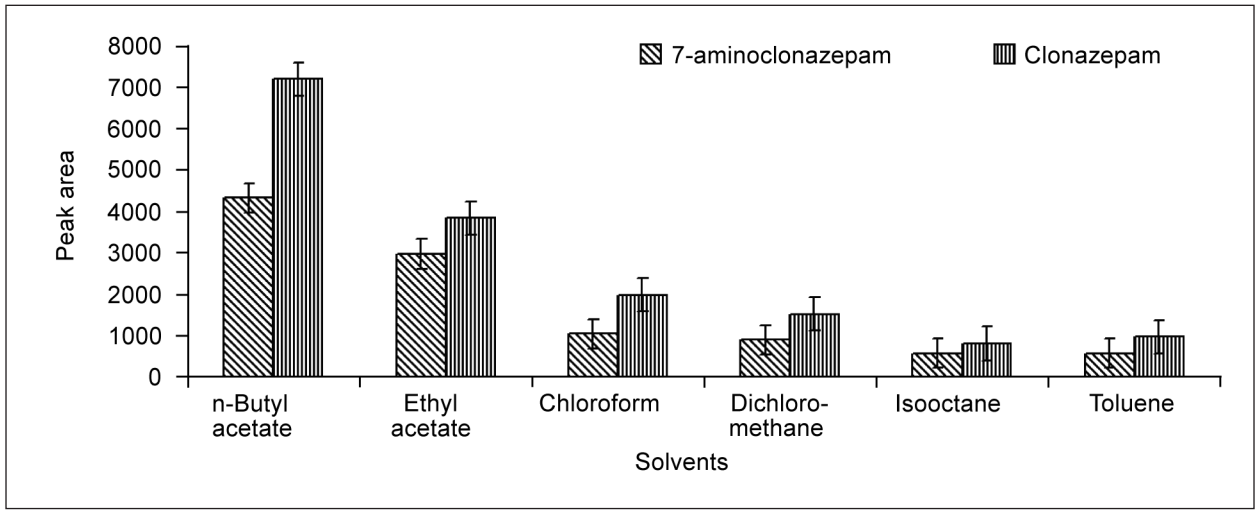

Fig. 3. Effect of solvent nature on the extraction efficiency of clonazepam and 7-aminoclonazepam (n = 5)

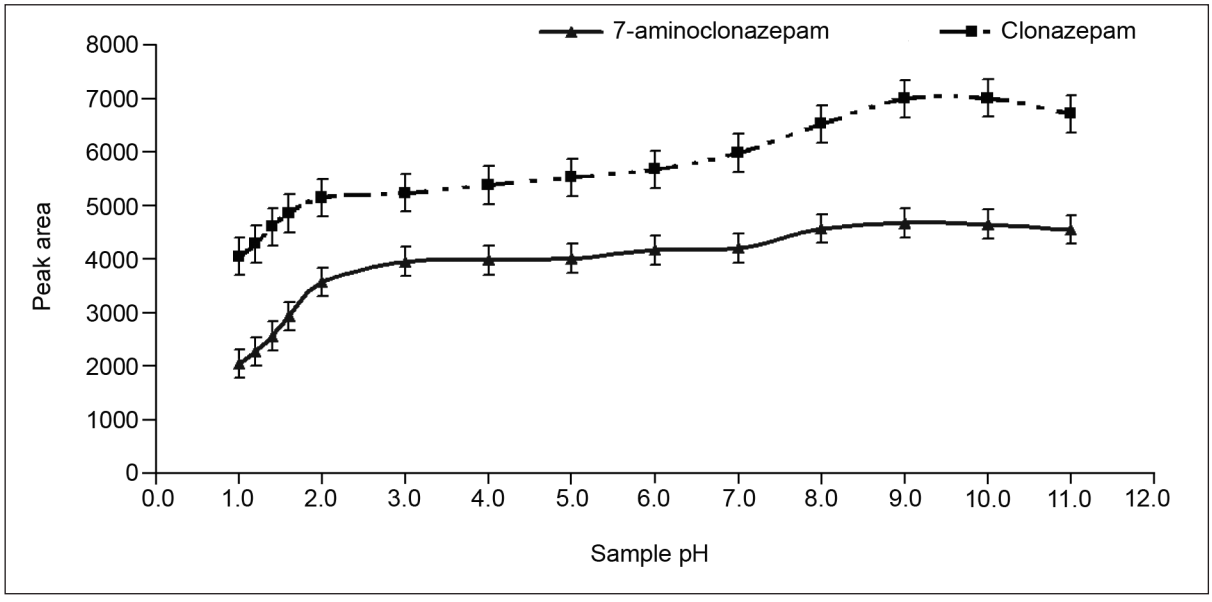

Fig. 4. Effect of sample pH on the extraction efficiency of clonazepam and 7-aminoclonazepam (n = 5)

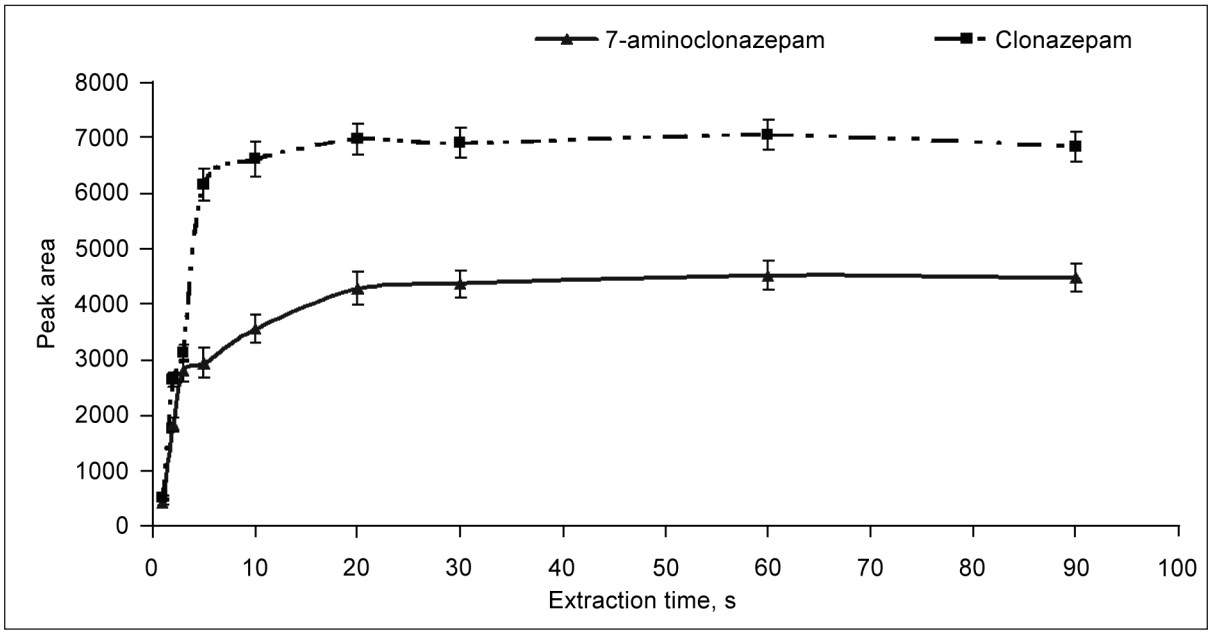

Fig. 5. Effect of extraction time on the extraction efficiency of clonazepam and 7-aminoclonazepam (n = 5)

reached a maximum by the extraction from alkaline sample solutions (pH ≥ 9.0). The extraction efficiency from less alkaline samples decreased likely due to the protonation of the analytes. Based on these results, the pH value of 9.5 was selected for further studies.

Optimization of extraction time

The influence of extraction time was investigated in the range of 2–90 seconds. The plots of peak area versus extraction time (Fig. 5) show that the extracted amounts of both analytes dramatically increase with the extraction time in the range of 2–20 s and then stay almost constant until 90 s. Therefore, 20 s were selected as sufficient time for effective extraction of both analytes.

Optimization of derivatization procedure

Although the analytes can be determined by GC/NICI-MS without derivatization, several problems are usually encountered for the trace level analysis. Losses of analytes and peak tailing due to adsorption in the GC inlet device and / or an interaction of the analytes with active sites on the walls of the capillary column can be observed. As already mentioned above, MTBSTFA was found to be the most suitable reagent for the derivatization of analytes. In addition to the reduced

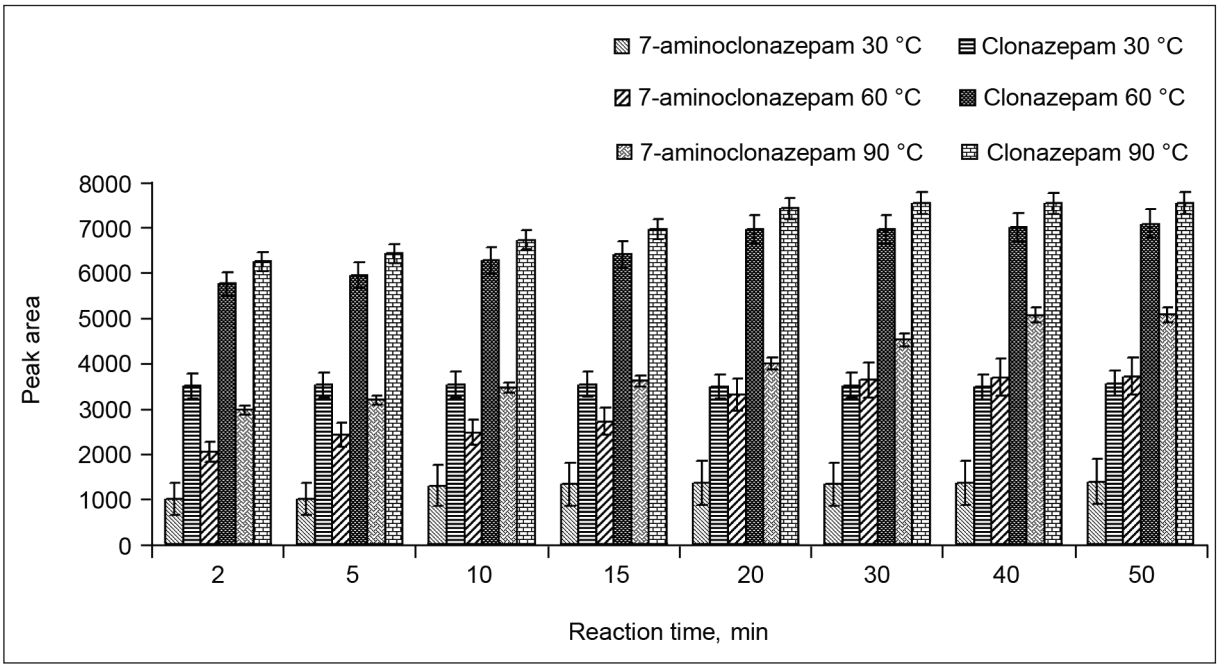

Fig. 6. Effect of reaction time and temperature on the derivatization efficiency of clonazepam and 7-aminoclonazepam (n = 5)

peak tailing and analyte adsorption effects, derivatization also enhances the detection sensitivity. For instance, if compared with non-derivatized analytes, the peak areas of derivatized ones increased in about 5 and 8 times for clonazepam and 7-aminoclonazepam, respectively.

In the next step of our research, the derivatization time and temperature were optimized. The reaction times of 2–50 min and temperatures of 30, 60 and 90 °C were examined. Fig. 6 compares the peak areas for clonazepam and 7-aminoclonazepam obtained under different derivatization conditions. The optimum reaction conditions for silylation by MTBSTFA were found at 90 °C for 40 min.

The selectivity of method

The selectivity was studied by analyzing six different blank samples and the matrix effect was assessed. The selectivity of the method was adequate with minimal matrix effect on all blank samples. No endogenous blood components were eluting at the retention time of the analytes.

The specificity of method

The specificity was determined by analyzing a standard solution mixture of commonly used illicit and licit drugs or their groups as well as their metabolites (opiates, amphetamines, cocaines, cannabinoids, methadone, diazepam, nordiazepam, oxazepam, bromazepam, temazepam, midazolam, alprazolam, flunitrazepam, lorazepam, triazolam, phenobarbital, amitriptyline, clomipramine, venlafaxine, citalopram, mirtazapine, ephedrine and ketamine) at a concentration of 1 000 ng mL^{-1}. Spiked blood samples with these substances (n = 6) at a concentration of 500 ng mL^{-1} were analyzed. The specificity study showed that blood concentration of 500 ng mL^{-1} of all drugs tested do not interfere with the accurate determination of clonazepam and 7-aminoclonazepam in whole blood.

Linearity

Calibration curves were obtained for clonazepam and 7-aminoclonazepam using a series of standard solutions in the concentration range of 5–200 ng mL^{-1}. Twelve replicate injections of standard at each concentration were performed. Calibration curves and the main parameters associated with them were studied using both peak areas and peak heights relative to the IS. Not very good results were achieved using peak areas without IS. Both tested analytes showed good linearity in whole blood with correlation coefficients between 0.999 and 0.995 and the RSD ranged between 2.83 and 3.16%.

The sensitivity of method

The limit of detection (LOD) and limit of quantification (LOQ) data were calculated on the basis of the extraction of twenty drug-free human blood samples (blood blanks) at a signal-to-noise ratio of 3 and 10, respectively. The LOD values of the clonazepam and 7-aminoclonazepam detection in whole blood were 1.0 and 1.4 ng mL^{-1}, respectively; LOQ values for determination of clonazepam and 7-aminoclonazepam were 2.5 and 3.2 ng mL^{-1}, respectively. They allowed the analysis of clonazepam and 7-aminoclonazepam in human blood samples at trace levels.

Recovery and precision

The recovery within-day and intermediate precision results are presented in Table 2. High recovery percentages show the

Table 2. **Recovery within-day (n = 6) and intermediate (n = 20) precision of the method for spiked drug-free whole blood samples**

Analyte	Mean concentration, ng mL^{-1}	Recovery, %	Within-day precision, RSD, %	Intermediate precision, RSD, %
Clonazepam	9.3/48.6/98.5/153.2	93.0/97.2/98.5/102.1	5.0/4.8/3.9/3.7	–
Clonazepam	11.5/45.3/89.7/148.2	115.0/90.6/98.7/98.8	–	7.0/6.4/5.5/4.1
7-amino-clonazepam	10.9/51.2/105.3/149.5	109.0/102.4/105.3/99.7	4.1/4.5/3.5/3.2	–
7-amino-clonazepam	10.5/50.3/99.2/151.4	105.0/100.6/99.2/100.9	–	6.7/5.9/4.1/3.9

applicability of the extraction system in spite of a low volume of organic LLE solvent (n-butyl acetate), low organic / aqueous phase ratio (1 : 1) and rapid LLE mixing procedure. The LLE recoveries obtained from QC whole blood samples at four concentrations of 10.0, 50.0, 100.0 and 150.0 ng mL^{-1} were 90.6–115.0 % and RSD values of within-day (repeatability) and time-different intermediate precision on the basis of peak area were less than 7.0%. Obtained results are comparable with those previously reported for tested analytes using sample preparation by LLE or SPE techniques at slightly basic (pH 9.0) or neutral (pH 6.0) conditions [10, 15].

Stability of the processed samples

The stability of the analytes in the injection solvent was studied to verify that compounds would not degrade over the course of an analysis. This was accomplished by extracting samples, storing and running them into the chromatographic system with a fresh standard curve on the following day. Reconstituted extracts were found to be stable at ambient temperature up to 24 h. The RSD values of a QC sample (medium concentration 100 ng mL^{-1}) at the twentieth repeatability for clonazepam and 7-aminoclonazepam at room temperature after 24 h were not significantly different; RSD values were 4.2 and 4.4%, respectively. The stability of samples stored in a –20 °C freezer was also determined. The compounds were found to be stable at –20 °C for thirty, sixty and ninety days with RSD for clonazepam and 7-aminoclonazepam lower than 5.9 and 7.6%, respectively. The clonazepam and 7-aminoclonazepam could be stored at –20 °C for ninety days, degradation of sample peak areas was less than 16 and 22%, respectively. These results are well fitting within acceptable criteria and indicate that the assay is robust under conditions likely to be encountered during sample handling.

Economics of analysis

Costs of single sample processing are lower compared to other available methods including SPE [9, 15]. On an average, a single chemist could prepare around 100 samples in the 8-h working period and the GC/NICI-MS output was 300 samples in 24 h cycle. The solvent exposure to the chemists was also significantly low, which establishes operational safety.

CONCLUSIONS

A fast and efficient method for the identification and quantitation of clonazepam and 7-aminoclonazepam in human whole blood is proposed and evaluated. This method is the fastest among the others reported up to now and it is suitable for toxicological determination of both analytes by the fast GC/NICI-MS technique. Sample preparation conditions including selection of the solvent, pH values, extraction duration of LLE, derivatization conditions, reaction temperature and duration were also optimized, and finally the stability of samples was evaluated. Sensitive and specific NICI-MS detection combined with fast GC resulted in sharp and symmetric peak shapes of the target analytes, while maintaining sufficient resolution. The developed method can be applied in routine toxicological analysis during the investigation of both clinical and forensic cases.

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GREITAS IR LABAI JAUTRUS KLONAZEPAMO IR 7-AMINOKLONAZEPAMO NUSTATYMAS KRAUJYJE DUJŲ CHROMATOGRAFIJOS IR MASIŲ SPEKTROMETRIJOS NEIGIAMOS CHEMINĖS JONIZACIJOS METODU

S a n t r a u k a

Klonazepamą ir jo pagrindinį 7-aminoklonazepamo metabolitą galima nustatyti dujų chromatografijos ir masių spektrometrijos metodu naudojant jonizacijos šaltinį – neigiamą cheminę jonizaciją (GC/NICI-MS). Gaunami patikimi rezultatai ir puiki galimybė nustatyti šiuos junginius kraujyje. Nustatyta skysčių-skysčių ekstrakcijos ir derivatizacijos efektyvumo parametrai: ekstrahento pobūdis, mėginio pH, ekstrakcijos laikas, derivatizacijos laikas ir temperatūra buvo optimizuoti. Analitės, ekstrahuojamos naudojant n-butilo acetatą, derivatizuojamos su N-metil-N-(tert-butildimetilsilil)trifluoroacetamidu (MTBSTFA). Klonazepamo ir 7-aminoklonazepamo kraujo mėginio aptikimo ribos buvo 1,0 ir 1,4 ng mL^{-1}, kalibravimo kreivės yra tiesinės koncentracijos diapazone 5,0–200,0 ng mL^{-1}, koreliacijos koeficientas $r^2 > 0{,}995$. Tyrimo metu naudojamų kokybės kontrolės mėginių santykiniai standartiniai nuokrypiai buvo mažesni nei 7,0 % (n = 20). Gauti rezultatai parodė, kad GC/NICI-MS metodas yra greitas, tikslus, jautrus ir selektyvus.