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Research Article

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Determination of Giazepam Aminobenzophenones by two –dimensional gas chromatography-mass spectrometry (GC-GC-MS) in urine

M. A. Savchenko[1] and G. P. Petyunin[2]

[1]Forensic Toxicological Department of the Regional Office of Forensic Medical Examination, Gruzinenka St. 11, Cherkassy, Ukraine
[2]Kharkiv medical academy of postgraduate education, Korchagincev St. 58, Kharkiv, Ukraine

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ABSTRACT

In this study, an evaluation of suitable extraction and clean up techniques for benzophenones of gidazepam in urine samples was done. Solvent extraction methods were compared with solid-phase extraction using Bond Elut Certify. For end-step detection method two–dimensional gas chromatography-mass spectrometry (GC-GC/MS) were evaluated. Presence of aminocarboxibromobenzophenone in the urine was an indicator of the subject being a probable abuser of gidazepam.

Key words: gidazepam, aminobenzophenones, solid-phase extraction, two-dimensional gas chromatography, mass spectrometry.

INTRODUCTION

Gidazepam (1-(hydrazinocarbonyl)-methyl-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-on) (1) Fig. 1, also known as Diamidazepam, Hydazepam, belongs to group of derivatives 1,4-benzodiazepine, developed in Ukraine [1, 2]. It is a drug which is an atypical benzodiazepine derivative. An effect of "day" tranquilizer and selective anxiolytic has. Gidazepam possesses an original spectrum of pharmacological activity, combining anxiolytic and activating actions with antidepressant components. The maximum effect is reached within 1-4 hours, with the subsequent gradual easing. The main feature and advantage of Gidazepam before other drugs from anxiolytic group is absence sedative and muscle relaxic actions, ability to optimise training and operative activity and also low toxicity.

As well as others benzodiazepines it strengthens and prolongs effects of drugs of abuse such as opiates and amphetamines. However Gidazepam not included under schedules of controlled substances.

Gidazepam is a prodrug for its active metabolite 7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepine-2-one (3) Fig. 1. The metabolism of gidazepam proceeds quickly already at administration, because in a human body are found out only its metabolites (2) and (3) [1,3,4] Fig. 1.

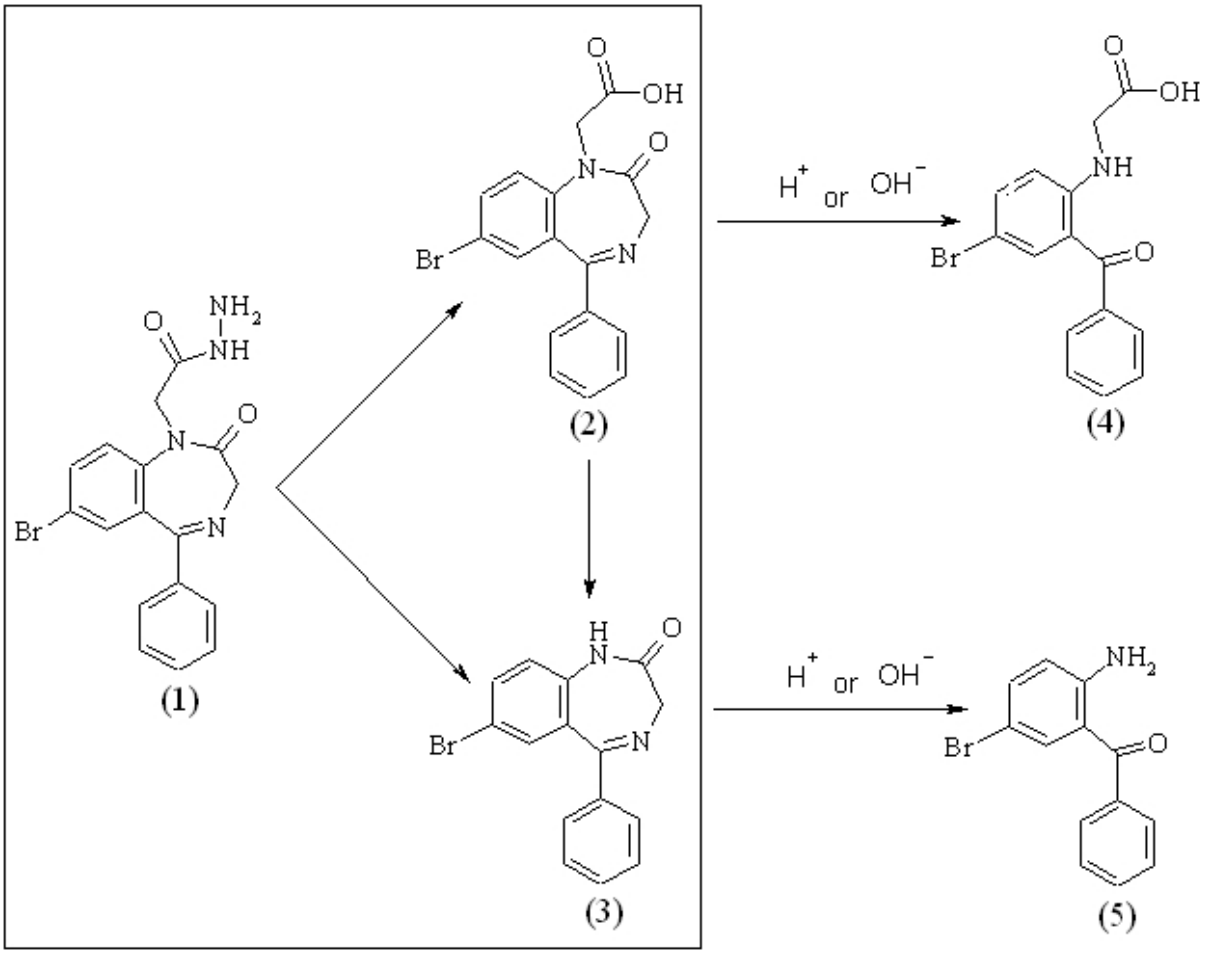

Fig. 1: Gidazepam, its metabolites, and their aminobenzophenones

As it is known, the first stage of toxicological screening on derivatives 1,4-benzodiazepine, is their hydrolysis and them метаболитов to corresponding benzophenones. The studying of acid and alkaline hydrolysis of Gidazepam and its metabolites [4, 5], has shown the big efficiency of alkaline hydrolysis. Products of this hydrolysis were aminobromobenzophenone (ABB (5)) and aminocarboxibromobenzophenone (ACBB (4)) Fig. 1. The last compound (ACBB) is specific benzophenone only for gidazepam, present at samples of urine and can be used as a marker of the application of it. At the same time, we showed that some benzodiazepine drugs can give benzophenones, which are similar to benzophenones of gidazepam that complicates their identification by TLC method [4, 6].

This paper describes the identification of aminobenzophenones of gidazepam metabolites in urine samples by two-dimensional (heart cutting) gas chromatography with quadrupole mass-selective detector (GC-GC/MS).

EXPERIMENTAL SECTION

All solvents were of analytical grade (Merck, Darmstadt, Germany). Aminobromobenzophenone was purchased from Subsidiary Liability Company ”InterChem” (Odessa, Ukraine). Aminocarboxymethylbrombenzophenone was prepare from gidazepam by hydrolysis in 5% (w/w) aqueous solution of sodium hydroxide for 20 min, and recrystallized from ethanol. Other aminobenzophenones were prepare by acid hydrolysis from corresponding benzodiazepines(ACB from oxazepam, ABCB from fenazepam, MCB from diazepam, ANB from nitrazepam, ACNB from clonazepam) and purified by preparative TLC (SiO_2) in benzene, as mobile phases. Diazomethane was prepare from nitrosomethylurea and absorbed in ethyl acetate. Phosphate buffer (0.1M) was prepared by dissolving 6.81 g of potassium dihydrogen phosphate into 450 mL of distilled water, adjusting the pH to 6.0 (±0.1) with 1.0M potassium hydroxide, and making the total volume up to 500 mL with distilled water. The 1.0M acetic acid solution was prepared by mixing 5,72 mL of glacial acetic acid with 100 mL of distilled water.

Bond Elut Certify columns (130 mg sorbent mass/10 mL column volume) were purchased from Agilent Technologies (Palo Alto, CA, USA).

The stock solutions of 1 mg/mL each aminobenzophenones, were prepare in methanol. A working (100 µg/mL) solution was prepared by dissolving 1.0 mL of the stock solution in 10 mL of methanol. The blank urine, spiked with aminobenzophenones, was prepared by added to urine (1.0 mL) after hydrolysis 20 µL working solution.

To 1mL urine in a screw-capped test-tube add 0.15 mL of 40% (w/w) sodium hydroxide. Screw the cap on to the test-tube and allow it is to stand in a boiling water-bath for 20 min. After hydrolysis, cool the test-tube and adjust pH to 2-3 for liquid-liquid extraction (LLE) or to pH 6-7 for solid-phase extraction (SPE) with 2.0M hydrochloric acid.

To 5 mL of the acidified sample add extraction solvent (1-chlorbutane-ethylacetate (9:1v/v)). Vortex for 5 min and centrifuge for 10 min at 2000g. Transfer the organic layer in to the clean heart-shaped flask and evaporate to dryness under a stream of nitrogen or under vacuum.

To the neutralized sample add 3mL of 0.1M phosphate buffer pH 6.0, vortex 1 min and centrifuge if necessary. Before extraction, SPE column conditioned with 2 mL of methanol and 2 mL of phosphate buffer[7]. Transfer the diluted mixture to the SPE column without vacuum. Wash the column without vacuum by passing through it sequentially: **a**. 1mL phosphate buffer pH 6.0; **b.** 1 mL mixture phosphate buffer (pH 6.0)-methanol (8:2); **c.** 1 mL of 1.0M acetic acid. Dry the SPE column for 2 min under maximal vacuum and dry the wall of the column by filter paper. Wash the column under light vacuum with 1 mL hexane. Dry the SPE column for 5 min under maximal vacuum. Elute the aminobenzophenones without vacuum with 1.5 mL mixture of hexane-ethyl acetate (8:2 v/v). Collect eluate in to the clean heart-shaped flask and evaporate to dryness under a stream of nitrogen, or under vacuum.

For derivatization to the extract in heart-shaped flask add 0.5 mL solution of diazomethane in ethylacetate, allow it to stand at room temperature for 10 min. Evaporate ethylacetate under a stream of nitrogen or under vacuum. Reconstitute the residue in 100 µL of methanol.

The GC-MS system was Agilent 6890N(GC)/5973N(MSD)/FID with microfluidic Deans switch between columns, from Agilent Technologies. First column: HP-5ms, (30 m × 0.25 mm i.d., film thickness 0.25 µm) was connected from the inlet to the Capillary Flow Technology (CFT) tee. Second column: DB-17ms, (30 m × 0.25 mm i.d., film thickness 0.25 µm), was connected from the (CFT) tee, to the MS. Uncoated, deactivated silica retention gap was connected from the Deans switch to the FID. Flow of carrier gas out first column could switch between FID, or second column.

Injection temperature: 250 ^{0}C, initial pressure held 26.00 psi for 2 min, then increased at a rate of 4 psi/min to 40.00 psi, then increased at a rate of 1.4 psi/min to 50.22 psi, and held at this pressure for 1.17 min, then increased at a rate of 4 psi/min to 40.00 psi, then dropped at a rate of 60.00 psi/min to 0.50 psi, and held at this pressure to the end of the run. The injector was set in the splitless mode and injection volume was 1uL with auto injector 7683.

Initial pressure of pneumatic control module (PCM) held 19,35 psi for 2 min, then increased at a rate of 4 psi/min to 30,40 psi, then increased at a rate of 1.4 psi/min to 38,72 psi, and held at this pressure for to the end of the run. ABB cut time start/end: 12.98/13.08 min, ACBB cut time start/end: 14.94/15.09 min.

Initial temperature of oven held 70 ^{0}C, for 2 min, then increased at a rate of 45 ^{0}C/min to 210 ^{0}C, then increased at a rate of 15 ^{0}C/min to 320 ^{0}C, and held at this final temperature for 7.56 min.

Mass selective detector (MSD) - transfer line temperature: 280 ^{0}C, ion source and quadrupole temperature were 230 ^{0}C and 150 ^{0}C respectively, electrons energy: 70 eV, the electron multiplier voltage set at 200 volts above the autotune voltage, mass range in total ion mode: 50-510 a.m.u., threshold: 110. FID - temperature: 250 0C, hydrogen flow: 30 mL/min, air flow: 400 mL/min, helium makeup flow: 25 mL/min.

The software used for data acquisition and manipulation was Enhanced MSD Chemstation version E.02.00.493.

Confirmation and identification aminobenzophenones based on retention time on both column and compare mass spectral characteristics with referents spectrums. Retention time windows on first column were between 0.10 (for ABB) and 0.20 (for ACBB) min. On second column for both compounds retention time windows was 0.20 min. In order to ensure accurate cut-times, spiked urine extract was run prior to each analysis to check the cut times. The matched quality values for the compared full mass spectra were higher then 80 %, and all measured ions with a relative intensity of more than 10 % in the reference spectrum, in spectrum of the analytes were presents. The relative intensities of the ions for both SIM, full scan modes, within the recommended tolerances [8]. Referents spectrums were recording from spiked urine analyzed under the same conditions.

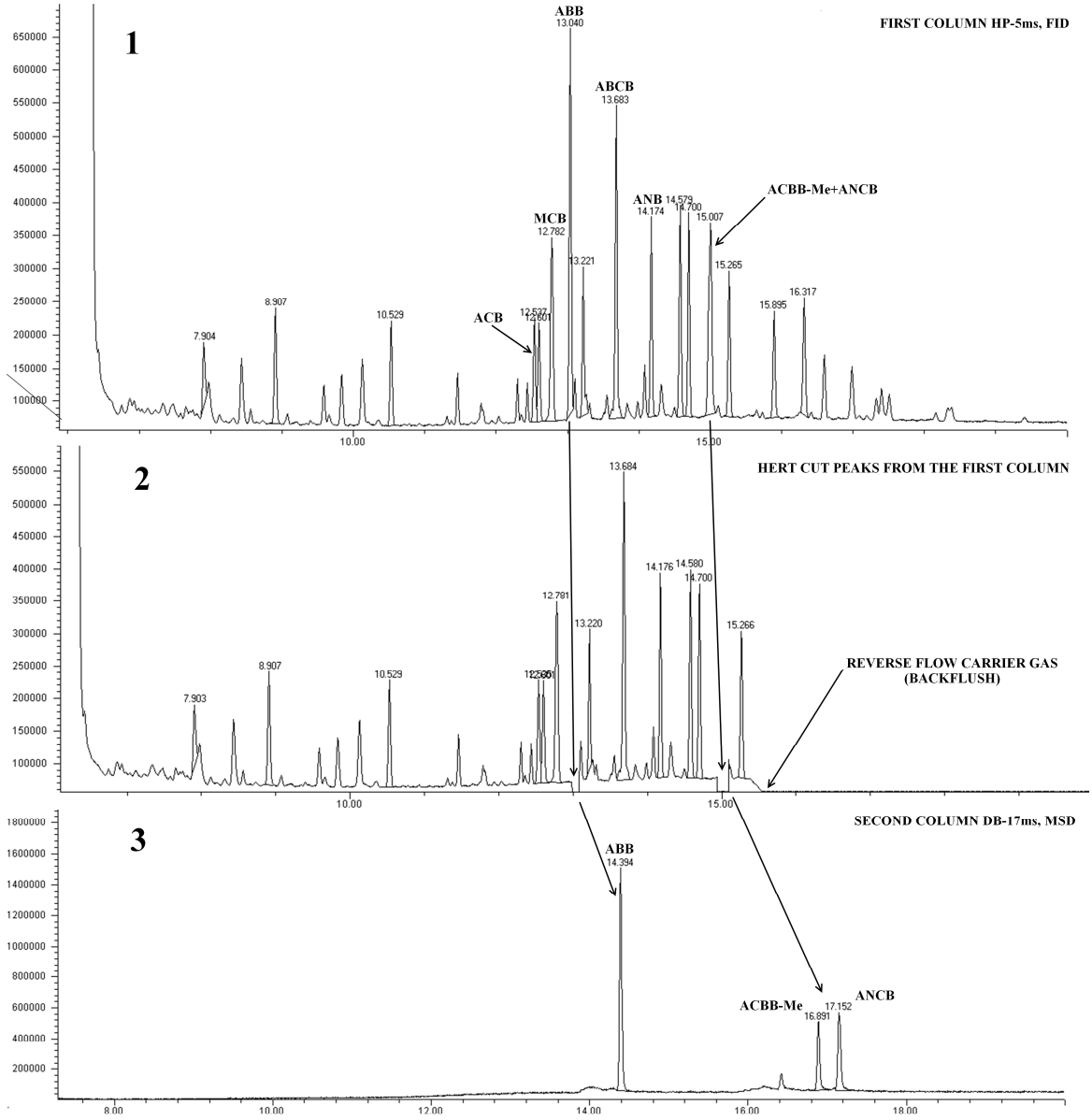

Fig. 2. Separate on single HP-5ms column (1), heart cutting and "backflushing" (2), separate on second DB-17ms column after heart cutting (3)

RESULTS AND DISCUSSION

Comparative research of processes extraction of gidazepam metabolites has shown that both methods - LLE and SPE have appeared effective enough. So, recovery of ABB and ACBB at LLE method have made 92 % and 89 %, and at SPE method – 96% and 98 %, accordingly.

In paper [9] we showed, what GC separation of ACBB can be performed only after transformation of the native compound to less polar derivatives. With that purpose we used a solution of diazomethane. With this reagent methylation complete at room temperature for 10 min. Additional, most clear chromatograms were obtained with derivatization diazomethane than silylation reagents.

Chromatographic separated was performed sequentially on two columns different polarity by GC-GC/MS. Upon injection, the ABB and ACBB separate on the first column. The Deans switch is time programmed to heart cut the elution time range of aminobenzophenones from first column onto the second column, where they additionally separated from the co-eluted compounds and into the MS. This technique offers several advantages. From GC, increase chromatographic resolution. So, when ACB, MCB, ABCB, ANB, ANCB with ABB and ACBB-Me examines, heart cutting on second column allow separate ACBB-Me and ANCB, which chromatographically overlap on column HP-

5ms. Sequentially separate on two different polarity columns, allows apply two retention time windows for a higher degree of conformation. Applying of double retention time windows particularly are useful when SIM mode performed. Signals from co-extracted matrix components were minimal when urine extracts analyzed. In general, second chromatogram has got more simple kind and from mass detector, more "clear" mass spectrums of ABB and methyl ether of ACBB (ACBB-Me) obtain Fig. 2, 3. In additional, at the end of ACBB-Me elution, the carrier gas in first column can be reversed to backflush unwanted heavy sample components out the split vent in the inlet, simultaneous with separating of analytes on second column.

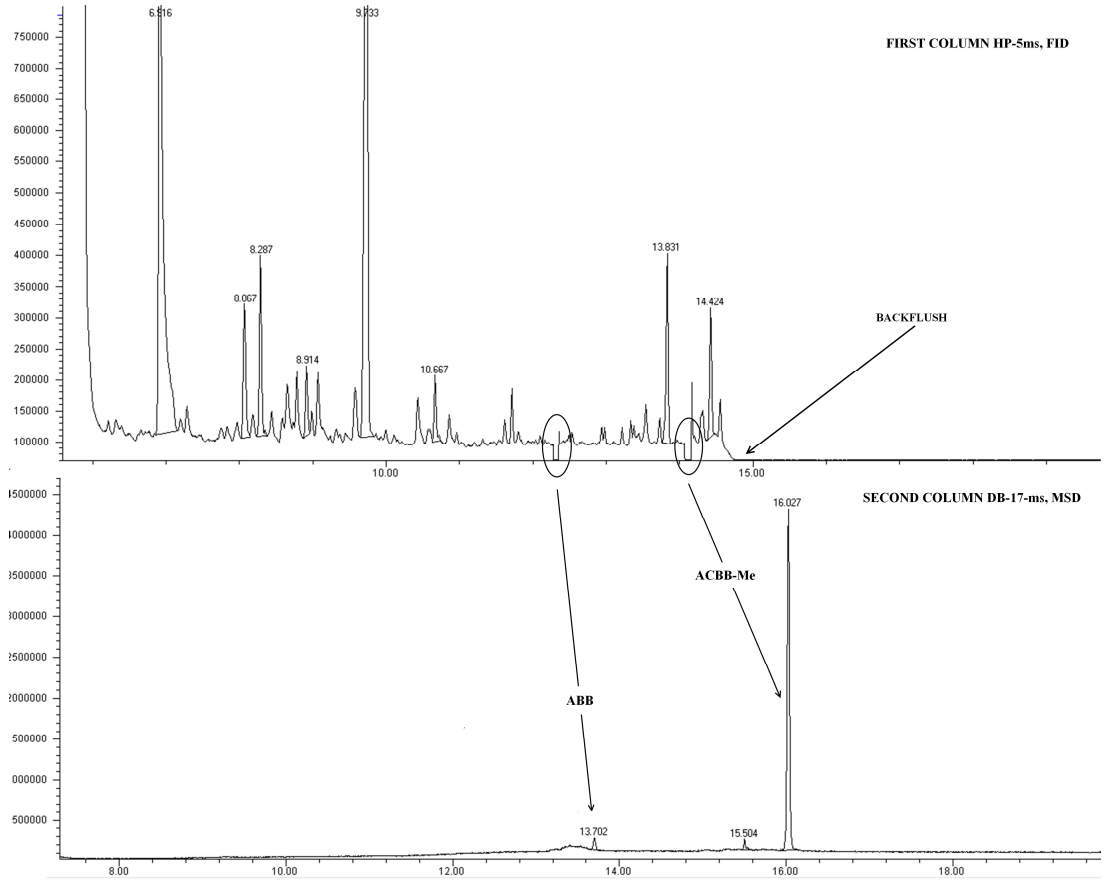

Fig. 3. 2-D chromatogram of urine extract. Second signals record as total ion chromatogram

In table 1. showed the m/z values of the ten ions, are listed in descending order of abundance, of the ABB and ACBB-Me, with a relative intensity more than 10 %. Spectrums were obtained after subtract of background and applied when identification performed. We choice five (for ABB) and six (for ACBB) ions for SIM mode. One ion was base, other qualifiers. For ABB, ions with m/z values 105, 167 и 195 more abundance that isotopic ions with m/z values 170, 172, 198 and 200, but ion chromatogram with those ions makes significant background interferences and did not use. As well as ABB, for ACBB-Me molecular ion with m/z 347 change to isotopic ion with m/z 349. Ions with m/z 210, 271 and 331 makes significant background interferences and did not use too.

Table 1. Mass spectral data of aminobenzophenones

Compound	*m/z* values of ions		
	Full mass spectra	SIM	
		base	qualifiers
ABB	**276 (999)M^+**, 274 (932), 275 (810), 277 (799), 77 (362), 195 (281), 105 (246), 198 (174), 200 (166), 98 (133), 167 (123), 91(104), 278 (102), 170 (98), 172 (98), 51 (97).	**276 (M^+)**	170, 172, 198, 200
ACBB-Me	288 (999), 290 (945), 210 (345), 91 (322), **347 (305)M^+**, 349 (304), 212 (298), 77 (267), 209 (227), 289 (174), 291 (146), 180 (127), 329 (100), 331 (98), 273 (99), 271 (95).	288	212, 273, 290, 329, **349 (M^+)**

None of the twenties blank urinary extracts from different sources showed false positive identification ABB and ACBB of both, full scan, SIM modes. Urine collected from 8-12 hours after single oral dose of 20 mg gidazepam was tested. ABB and ACBB were reliably identification in that urine for full mass spectra.

For both modes of operate MSD, the limit of detection (LOD), as minimal concentration of analytes which allows be identify with acceptable criteria, was found. When full scan operate, ABB and ACBB LOD was found as 70 and 50 ng/mL respectively. In that mode signal to noise rotations (S/N) for ABB – 30:1 (on mass m/z – 276), for ACBB (as ACBB-Me) - 43:1 (on mass m/z – 288). In SIM mode LOD of ABB and ACBB was found as 3 and 1 ng/mL, with S/N 14:1 and 18:1 respectively.

CONCLUSION

We developed a specific and sensitive method for testing gidazepam in urine by determining aminobromobenzophe-none and aminocarboxymethylbrombenzophe-none by GC-GC/MS. The LOD for all tested components was ≤3 ng/mL in 1.0 mL urine sample. For qualitative analysis was used both full scan ions and SIM modes. The analytes were well-separated with high recoveries when the appropriate SPE or LLE techniques were used. The proposed method, in which an LLE or SPE column and the GC–GC/MS system were used, can be used to determine gidaze-pam.

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