DEVELOPMENT OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF SEDATIVE-HYPNOTIC DRUGS IN BIOLOGICAL SAMPLES BY FAST GAS CHROMATOGRAPHY TECHNIQUE

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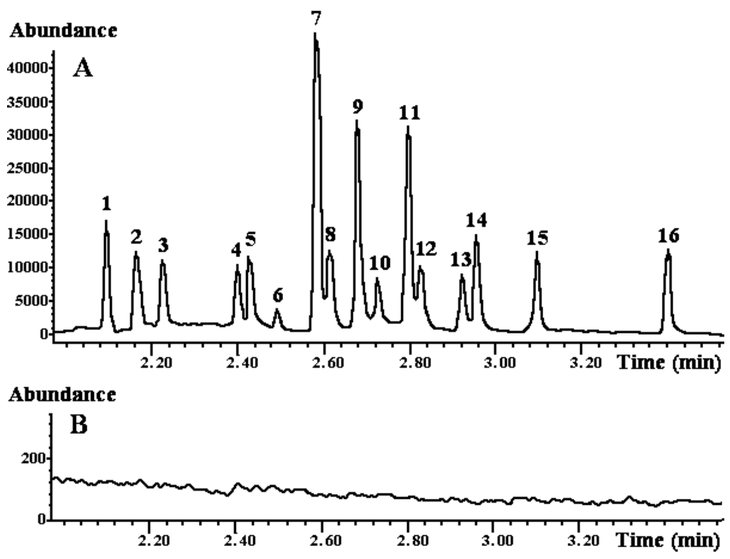
Benzodiazepines are an important class of drugs with a broad range of therapeutic effects, including sedative-hypnotic, anxiolytic, muscle-relaxant, and anticonvulsant [1]. For these reasons, the analysis of benzodiazepines is of great interest to forensic and clinical toxicologists. Therefore, it is very important to develop a rapid method for the determination of benzodiazepines. Gas chromatography combined with negative-ion chemical ionization mass spectrometry (GC/NICI-MS) improves the sensitivity and specificity of 15 benzodiazepines detection in blood, urine and significantly exceeds parameters achieved by traditional electron impact ionization mass spectrometric detection [2-5].

Many widely used screening and confirmation methods do not detect low concentrations of benzodiazepines in low-volume blood and urine samples. Therefore, the aim of my study was the development of a new sensitive and specific method based on GC/NICI-MS using a mixed-mode solid phase extraction for the identification and quantification of these drugs in blood and urine samples. To the best of my knowledge, this method has been used for the first time for the optimization of sample preparation at pH 1.0. Furthermore, the speed of the analytical separation was emphasized by modifying various GC/NICI-MS parameters. The fully validated method was applied for the quantification of several benzodiazepines in real blood and urine samples. Furthermore, the study was performed according to the recommendation [5].

Method development

The rapid chromatographic conditions were tested at: 220, 230, 240, *250* and 300 °C for injector temperature; 280, 290, *300* and 310 °C for detector temperature; 150, 170, *180* and 250 °C for initial column temperature; 290, 310, *325* and 340 °C for final column temperature; as well as 30, 40, *50* and 60 °C min^{-1} for the column temperature elevation rate; and finally 0.5, 1.0, 2.0, 3.0, *3.5* and 7.0 mL min^{-1} for the flow rate of carrier gas (results not shown). The optimal conditions are presented in bold (italic) and have been chosen based on the peak areas of analytes derivatized with N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) and those not derivatized, and based on their resolution.



The fast GC/NICI-MS separation of benzodiazepines was achieved within 3.9 min as shown in figure 1A.

Figure 1. SIM chromatograms were obtained from the analysis of benzodiazepines by GC/NICI-MS. (A) Chromatogram of a whole blood sample at pH 1.0 spiked with 20 ng mL^{-1} of each analyte after the washing steps of SPE. (B) Representative SIM chromatogram of a blank blood sample. The peak numbering refers to: (1) internal standard; (2) diazepam; (3) nordiazepam-TBDMS; (4) midazolam; (5) flunitrazepam; (6) bromazepam-TBDMS; (7) oxazepam-2TBDMS; (8) nitrazepam-TBDMS; (9) temazepam-TBDMS; (10) 7-aminoclonazepam-TBDMS; (11) lorazepam-2TBDMS; (12) clonazepam-TBDMS; (13) alprazolam; (14) α-OH-midazolam-TBDMS; (15) triazolam; (16) α-OH-alprazolam-TBDMS.

The figure was obtained for the first time [5].

For more information

1. This study was published in my doctoral dissertation, the license agreement no. VU-ETD-4 (10-04-2015)

2. N. Karlonas, A. Padarauskas, A. Ramanavicius, A. Ramanaviciene. Journal of Separation Science 36(8), 1437-1445 (2013)

Development of solid phase extraction

The adsorption-related behavior of benzodiazepines was similar. When the acidic solution of pH 1.0 was used, all analytes were adsorbed on the column due to ion-exchange interactions. All analytes have basic pK_a values in the range from 1.2 to 6.5 [6]. All analytes were eluted with MeOH containing 5 % of NH_4OH. When using a neutral or basic buffer of pH 7.0 or 9.0 respectively, analytes were adsorbed onto the polymeric sorbent in the column due to hydrophilic-lipophilic interactions and were eluted with pure MeOH. The extraction efficiency at pH 7.0 was insufficient for midazolam and triazolam. These analytes, with pK_a values 6.2 and 6.5, respectively, were not completely retained under neutral conditions due to lower hydrophobicity than at basic pH. Typical extraction efficiencies of 15 benzodiazepines at pH 1.0 and pH 9.0 are shown in Fig. 2.

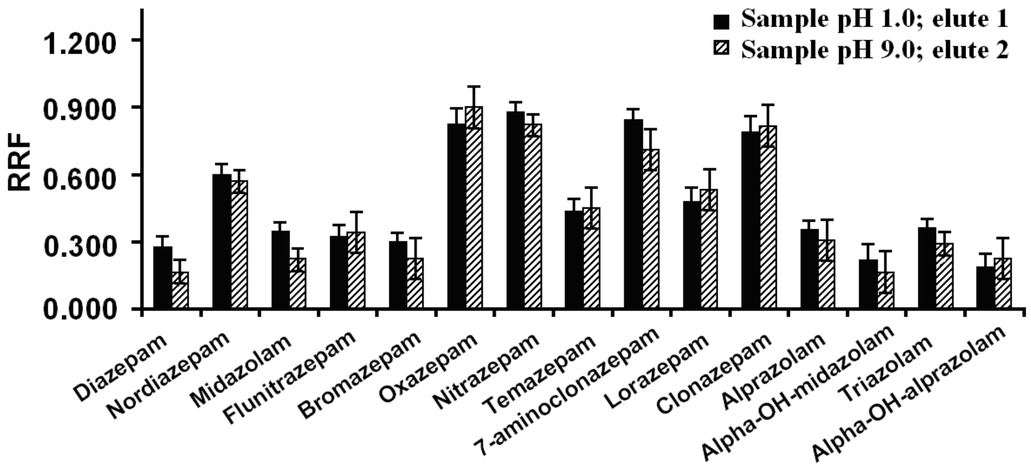


Figure 2. Extraction efficiency of 15 benzodiazepines at two pH values.

Elute 1 – MeOH containing 5 % (v/v) NH_4OH (n=5). Elute 2 – MeOH (n=5).
The figure was obtained for the first time [5].

The adsorption behavior of benzodiazepines on the SPE sorbent can be explained by the protonation/deprotonation of the analyte in the sample solution. All analytes, which are in the non-ionic form at pH 9.0 in a basic buffer, were adsorbed by reversed-phase interactions. In contrast, analytes which are in the cationic form in an acidic solution interacted with Oasis MCX specifically through ionic interactions. Additionally, benzodiazepines, which were retained by an ion-exchange phase, could be washed with organic solvents in order to remove acidic and neutral interfering compounds through mixed-mode interactions. After this step, visibly cleaner extracts were obtained in comparison with those obtained using reversed-phase interactions.

Three wash steps were employed to remove the matrix and to get very clean extracts. First, acidified water (0.1 M HCl of pH 1.0) was used, followed by organic solvents. Different volumes (1.5 and 1.0 mL) of both 0.1 M HCl of pH 1.0 and ACN were tested. These reagents did not cause the loss of any benzodiazepines, but the matrix was not sufficiently removed from the sample. However, 1.5 mL of pure MeOH, EtOH, 1-PrOH, 1-BuOH, or ethyl acetate resulted in removal of some analytes and in effective removal of matrix from the samples. The results obtained using these solvents are presented in Fig. 3A. It was observed experimentally that 1-propanol (1-PrOH) was the best solvent to remove all matrix effects from the sample. Therefore, in this washing step, mixtures of acidified water (0.15 M HCl) and 1-PrOH with a 1-PrOH concentration ranging from 0 to 100 % in intervals of 10 % were tested. The optimal concentration of 1-PrOH to minimize loss of analytes at this stage was 60 %. It could be concluded that this washing step yielded significantly cleaner extracts.

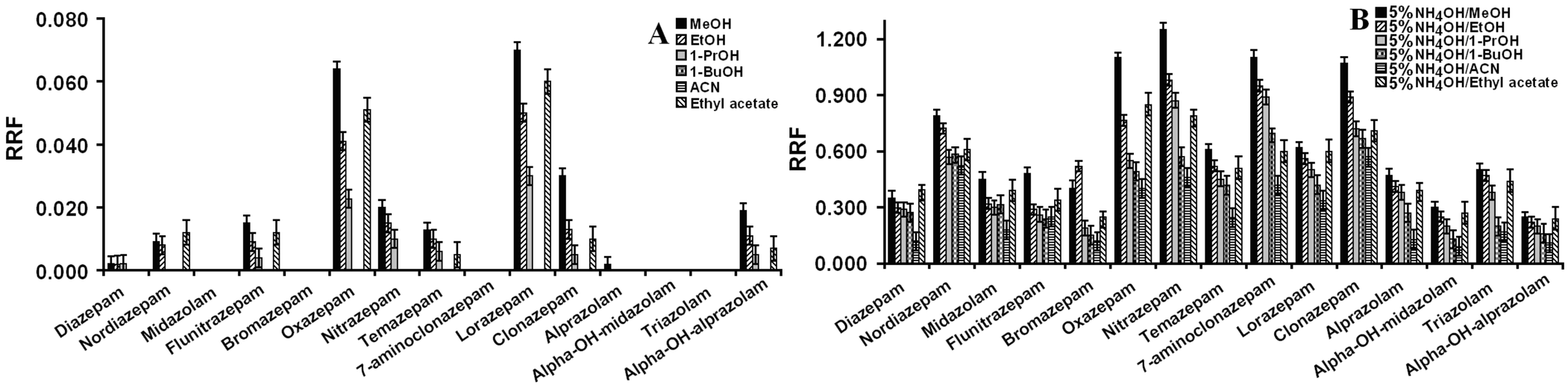


Figure 3. Extraction efficiency of 15 benzodiazepines at pH 1.0. A – eluted with organic solvents (n=5). B – eluted with solvents containing 5 % (v/v) NH_4OH (n=5).
The figure was obtained for the first time [5].

Finally, increasing volumes of 5 % NH_4OH in MeOH, EtOH, 1-PrOH, 1-BuOH, ACN, or ethyl acetate over the range from 0.25 mL to 3.0 mL were investigated for elution of analytes. For the elution of all compounds, a volume of 2.0 mL was determined to be the most effective. The results obtained using different solvents containing 5 % NH_4OH are summarised in Fig. 3B. A volume of 2.0 mL of MeOH containing 5 % NH_4OH was found to be the optimal solvent combination suitable for complete elution from a column. Therefore, it was possible to completely separate all analytes and interference compounds from whole blood using the described method (Fig. 1A).

Conclusion

The developed method allows specific and sensitive determination of benzodiazepines and their metabolites in blood and urine by fast GC/NICI-MS after SPE using a polymeric type sorbent with ion-exchange and silylation agent. The solvents used for washing and elution, the derivatization temperature, and duration for sample preparation at pH 1.0 were determined. The presented method has several advantages: elimination of interferences, low-volume of samples, a multi-residue analysis, and very fast chromatographic separation of analytes. My developed method shows high sensitivity and specificity towards analytes present in blood and urine samples.

References

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