SAS Journal of Medicine ISSN 2454-5112
SAS J. Med., Volume-3; Issue-4 (Apr, 2017); p-72-75
Available online at http://sassociety.com/sasjm/

Original Research Article

Development of HPLC Methods in Identification and Quantification of Some Benzodiazepines (Diazepam, Oxazepam, Clonazepam, Flunitrazepam and Nitrazepam)

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Abstract: The developed HPLC method was validated in the identification and quantification of diazepam, oxazepam, clonazepam, flunitrazepam and nitrazepam (benzodiazepines). These analyses were performed using an HPLC system with a diode array detector (λ =254nm), a Lichrospher RP 18 column having a 5µm internal diameter, a 10 mM potassium phosphate buffer (pH 2.5), drop-wise addition of phosphoric acid as well as using methanol-acetonitrile (10:27, v/v) as mobile phase having a flow rate of 1ml min-1 and an injection volume of 20 µl. Confirmation of using the instrument was done with identification of caffeine prepared standards which showed good linearity hence validating the method and proving the instrument was suitable for the carrying out the project. In analyzing these benzodiazepines, the isocratic elution was used which gave good peaks but took longer retention times to elute especially the diazepam which eluted further away taking the longest retention time. The gradient elution was introduced where changing the mobile phase to 33 after 15 minutes and then to 60 gave best peak resolution amongst the four close benzodiazepines as well as making the diazepam take a shorter retention time to elute hence giving the best peak separation and identification of these benzodiazepines. The elements of validation were performed on two selected benzodiazepines (nitrazepam and diazepam) where their standard preparations gave correlation values of 0.99626 and 0.99978 respectively indicating a good linearity of all the points hence confirm that the method is valid with their LOD being 8ppm and 2 ppm as well as LOQ being 26ppm and 6 ppm respectively. It can be conclude that, this method proved to give good separation of peaks, shorter retention times for peak elution, accurate, precise results as well as good linearity and so, it is recommended for the identification and quantification of most drugs.
Keywords: HPLC method, Benzodiazepines, Diazepam, Oxazepam, Clonazepam, Flunitrazepam, Nitrazepam.

INTRODUCTION

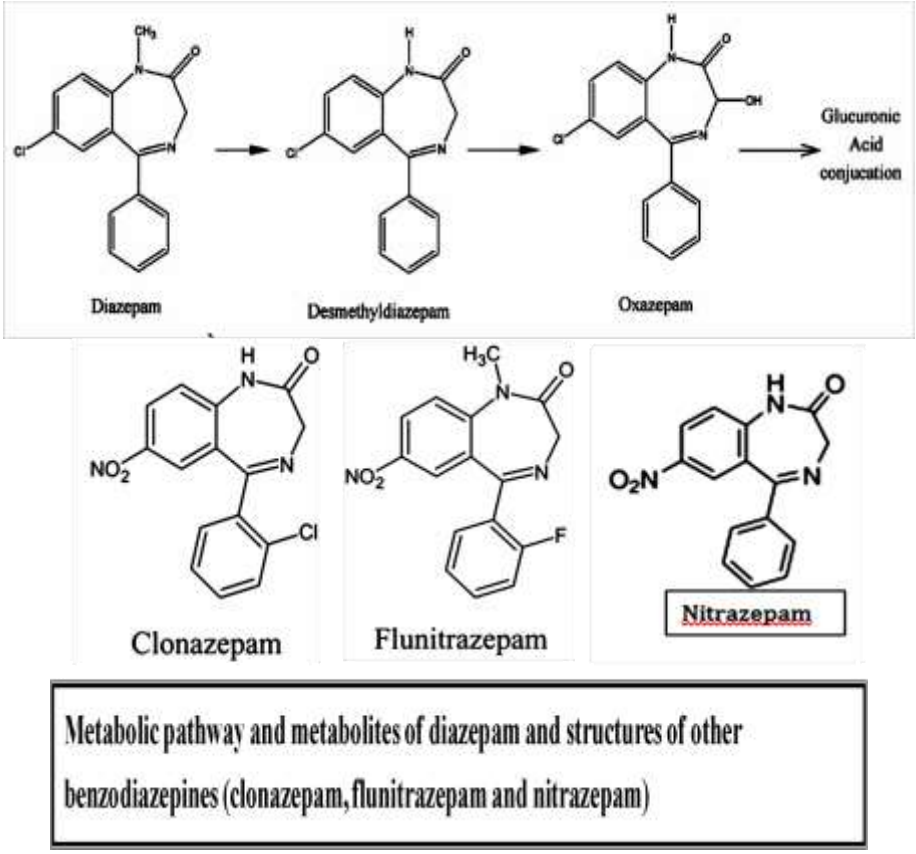
Classical benzodiazepines drugs have been of great importance in the clinical pharmacology and anaesthesia over a long period of time. They are used for anxiety and sedation (hypnosis) with an occasional use for general anaesthesia and this has developed a variety of adverse side effects [1]. Their use is recommended to be in a short time basis as prolonged exposures to patients may worsen the adverse effects coupled with development of drug dependency. The drugs due to uneven efficacy and affinity to GABA receptors they act in all receptor binding sites, and this subtype unselectively is usually characterised by a variety of clinical effects. This may lead to patients developing typical side effects especially in cases of prolonged drug use as the classical benzodiazepines exhibits both agonistic and inverse agonistic effects. Therefore classical benzodiazepines when administered to patients they do not show any demarcation between its anxiolytic and sedation effects but only differentiated by dose levels.

They usually show binding abilities to all GABAa receptor subtypes and due to undetermined efficacy levels in these binding sites, the drugs produces a dose dependent effects of anxiolytic, sedation, and even at elevated concentration there is general anaesthetic effects. Due to this unreliable properties of classical benzodiazepines, there is need for development of compounds with similar properties as classical benzodiazepines but with selective and high efficacies to specific GABAa receptor subtypes (binding sites), to produce a required clinical anaesthesiology effect. These novel agents will be useful in a management of chronic pain, depression, sedation and anxiety [1]. Benzodiazepines bind to all alpha subtypes of GABAa receptors, and they should be used in short term basis to avoid occurrences of

rebounds and withdrawal dependence characterised by epileptic fits and psychosis.

Benzodiazepines have a wide range of therapeutic effects such as sedative-hypnotic, anxiolytic, muscle-relaxant, and anticonvulsant making it an essential class of drug [2] but today as a result of being frequently prescribed , they have an increasing potential for addiction and abuse [3]. In forensic investigation the various specimens analyzed for the presence of drugs are urine, blood, vitreous humour, hair, nails, breast milk, meconium, sweat, saliva, stomach content as well as organs (liver, kidney, spleen, lungs) etc. The benzodiazepines investigated are those of the three ringed structures and are longer acting requiring higher dosages with slow metabolism and break down to biologically active compounds [4]. Benzodiazepines are known to respond differently to the various analytical methods due the differences in their lipophilicity, basicity and chemical reactivity [5]. Many benzodiazepines are known to form active metabolites with similar pharmacological properties in which there is lack of of specificity in distinguishing them from parent drug but nowadays several methods are being developed to target this issue. Of all the available methods used for the identification and quantification of benzodiazepines, HPLC is the chromatographic method of choice which is used for polar and thermally labile compounds (which is the case with benzodiazepines) with mobile phase modification to improve resolution and better results [6].

The aim of our study to develop suitable HPLC methods in identifying and quantifying the various benzodiazepines under study. Also, validation of this method through elements such as accuracy, linearity, sensitivity, precision, limit of detection and limit of quantification.



Metabolic pathway and metabolites of diazepam and structures of other benzodiazepines (clonazepam, flunitrazepam and nitrazepam)

MATERIALS AND METHODS

Materials

10mmol L^{-1} Potassium Phosphate buffer, Acetonitrile, methanol and Benzodiazepines. HPLC with a diode array detector at 254nm wavelength.

Methods

The mixture of the buffer and the acetonitrile/methanol was made in the ratio 65:35 respectively where the acetonitrile/methanol percentage in the mobile phase was varied from 20 to 60 including the 10mmol L^{-1} phosphate buffer. This was known as gradient elution where the methanol addition was for better peak resolution with all the 5 benzodiazepines identified as seen in figure 1.

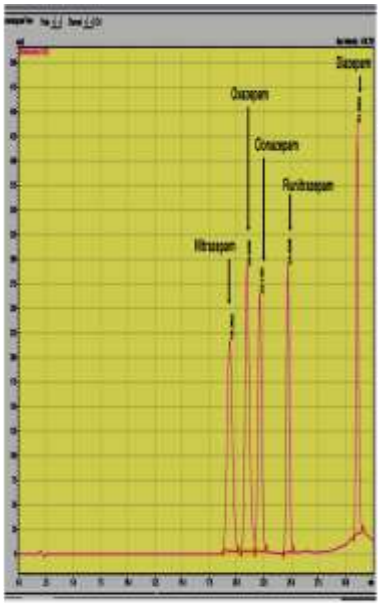


Figure 1. The identified peaks for the five benzodiazepines.

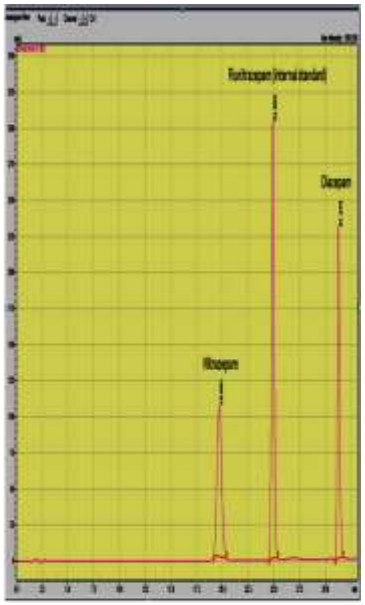


Figure 2. peaks for the three chosen benzodiazepines (nitrazepam, flunitrazepam and diazepam)

For quantification, Nitrazepam, flunitrazepan, diazepam were all mixed together, where the flunitrazepam acts like an internal standard and the peaks obtained are seen on figure 2.

RESULTS AND DISCUSSION

Using the HPLC to analyze the standards of each of the benzodiazepines, the retention times for each of them were obtained separately which served very useful for their identification when all of them were mixed together and analysed. There are some limitations, which exist in using chromatography for identification such as; identification of previously unknown substances or metabolites may become frequent, difficulties in detecting the escape of two compounds from the tubing at same time (coelution), its complex operation as well as the speed of the process which lowers the sensitivity to some compounds. Also, the HPLC have beads made of different materials found in the column where when mixtures are forced to pass through the column, the chemicals bind to the beads before being released, which if strongly bound, it will not be released hence hindering the measurement of the solution exiting the column.

Performing the gradient elution by varying the mobile phase between 20 to 60 gave the best peak resolution at a ratio of 67:33 for the phosphate buffer and acetonitrile+methanol respectively. After about 30 minutes the elution was changed to be in the ratio 40:60 in order to minimize the amount of time taken for the diazepam to elute.

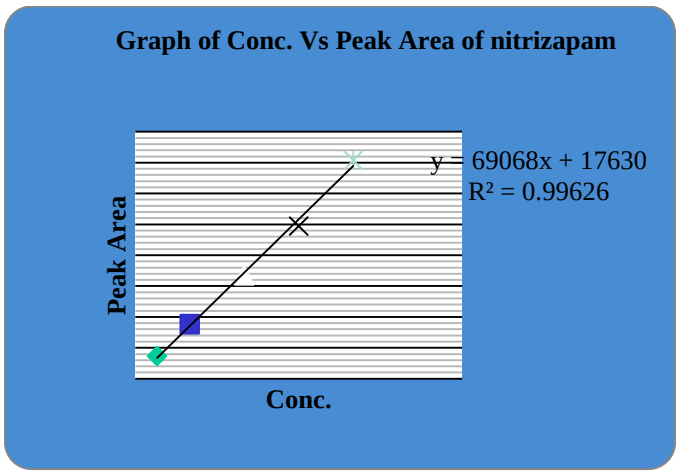


Fig-3: Concentration vs peak area of nitrazepam

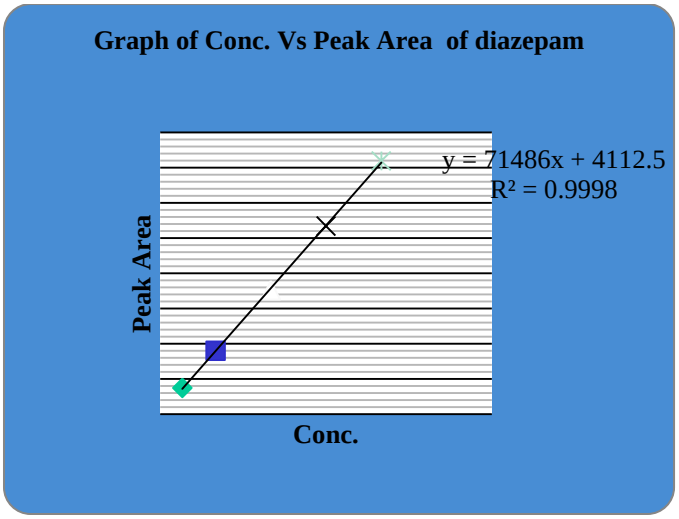


Fig-4: Concentration vs peak area of diazepam

Table 2: Showing LOD and LOQ of 2 Selected Benzo- diazepines (Nitrazepam and Diazepam).

Elements of Validation	Nitrazepam	Diazepam
Limit of Detection	8ppm	2ppm
Limit of Quantitation	26ppm	6ppm

CONCLUSIONS

The validation elements for the 2 selected benzodiazepines indicates that the results were accurate and precise as seen from the values of LOD and LOQ in the above table. The graphs for standard preparations of nitrazepam and diazepam as seen above, gave correlation values of 0.99626 and 0.99978 respectively indicating a good linearity of all the points hence confirm that the method is valid. It can be conclude that , this method can be best recommended for the identification and quantitation of most drugs since it has proven to give accurate, precise results as well a linearity.

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