ANALELE UNIVERSITATII BUCURESTI

Department of Physical Chemistry
4-12 Regina Elisabeta Blvd, District 3, Bucharest
phone: +40-21-3143508; fax: +40-21-3159249
*p*ISSN: 1220-871X *e*ISSN: 1844-0401

ARS DOCENDI PUBLISHING HOUSE
Sos. Panduri 90, District 5 Bucharest
phone/fax: +40-21-4102575
e-mail: arsdocendi@yahoo.com

THIN LAYER CHROMATOGRAPHIC SEPARATION OF BENZODIAZEPINE DERIVATES

G. Hancu *, Enikő Fülöp *, Aura Rusu *, Eleonora Mircia ** and Á. Gyéresi *

abstract: In this paper we describe the thin layer chromatographic separation of eight of the most frequently used benzodiazepine derivates (alprazolam, bromazepam, chlorazepate potassium, chlordiazepoxide, diazepam, nitrazepam, oxazepam) and their degradation products after acid hydrolysis. Our aim was not only to develop a simple, rapid and efficient method for their separation but also the optimization of the analytical conditions. Using silicagel GF254 as stationary phase and selecting six different mobile we succeeded in the separation of the studied benzodiazepines. Each benzodiazepine can be separated from the others by using an appropriate mobile phase. Any of the benzodiazepines can be identified by combining the results obtained with different mobile phases.

key words: benzodiazepines; thin layer chromatography; separation; acid hydrolisis

received: October 6, 2011 *accepted*: December 16, 2011

1. Introduction

Benzodiazepines (BZD) are currently by the far most important and most widely used anxiolytic drugs and some compounds are also as sedato-hypnotics, anticonvulsivants, muscle relaxants or anaesthetics [1]. To emphasize even more their importance we can mention the fact that in the 7th edition of the European Pharmacopeia (EPh 7) [2] there are 14 officinal BZD derivates.

Thin layer chromatography (TLC) is among various chromatographic methods, a comparatively simple, rapid and convenient method frequently used for identifying many pharmaceutical substances [2]. TLC is used in EPh 7 [3] for the separation of a particular BZD from its impurities or related compounds, but the methods described are less suitable for identification purposes.

Many papers dealing with the thin-layer chromatographic analysis of BZD have been published, but in most BZD and metabolites are first hydrolyzed to benzophenones, which are later identified by chromatography [4]. Actually only a few papers have described the separation of intact BZD for identification purposes [5-7].

* University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Gh Marinescu 38, 540139 Târgu Mureş, Romania. *corresponding author e-mail*: g_hancu@yahoo.com
** University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Organic Chemistry, Gh Marinescu 38, 540139 Târgu Mureş, Romania.

© 2011 *Analele Universităţii din Bucureşti*

In this study our aim was to develop and optimise a useful rapid and sensitive TLC method for identification and separation of eight of the most frequently used BZD derivates, and also to assess their degradation after an “in situ” acid hydrolysis on the chromatoplate.

2. Materials and method

Instrumentation

The TLC system consisted of a Camag Nanomat III automatic sampler, a Camag Linomat IV semiautomatic sampler (Camag, Switzerland), a 2-μl Hamilton microsyringe (Hamilton, USA), a Camag Normal Development Chamber and a Camag fluorescence inspection lamp (Camag, Switzerland). As stationary phase we used 20x20 cm pre-coated silicagel GF254 HPTLC glass plates (Merck, Germany).

Reagents

Benzodiazepines: alprazolam, bromazepam, chlorazepate potassium (Labormed, Romania), chlordiazepoxide, diazepam, nitrazepam, oxazepam (Terapia, Romania). All the BZDs were of pharmaceutical grade. We chose the eight most frequently used BZD derivates, each having different structural characteristics (Fig. 1).

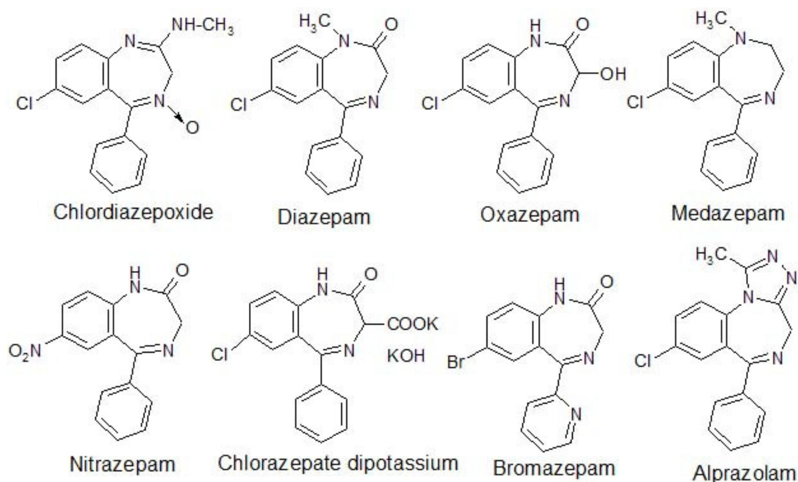

Fig. 1 *The structures of the eight studied BZD.*

Reagents: acetone, conc. ammonia, chloroform, ethyl acetate, isopropanol, conc. sulphuric acid (Chimopar, Romania), methanol, hexane (Merck, Germany).

Samples

The BZD samples were prepared at a concentration of 0.5% in methanol, except for chlorazepate dipotassium, which was dissolved in water and then diluted with methanol (1:1). Amounts of 0.5 μl were spotted on the chromatoplate.

Chromatographic procedure for in situ hydrolysis

Amounts of 0.5 µl of each BZD were spotted on the chromatoplate. Then 0.5 µl dilute sulphuric acid (10%) was placed over each spot; thereafter the plate was covered with a glass plate and kept for 15 minutes in an oven at 120^0C. The plate was cooled at room temperature and on each spot 0.5 µl concentrated ammonia solution (25%) was placed. The spots were dried by heating at 120^0C for 5 minutes.

Method

The chromatographic chambers were saturated with the mobile phase for 30 minutes. The plates were developed over a distance of 15 cm, dried in a stream of hot air, and examined first under UV radiation at wavelengths of 254 and 366 nm. Finally the plate was sprayed with Dragendorff reagent to visualize the spots and their Rf values were measured. All experiments were carried out at room temperature. Photographs of the chromatoplates were taken with a Nikon D-3100 camera; the camera being equipped with a UV filter.

3. Results and discussion

Optimization of the analytical method

The purpose of the method (separation of a multicomponent mixture), and the information about the samples (structure, polarity, solubility, stability) were important as initial hints for the choice of the chromatographic system, using the Stahl's triangle (Fig. 2). One corner of the triangle, which can rotate about its center, is set to the known the solubility of the sample, while the other two corners indicate the activity or polarity of the stationary and mobile phases [2,8].

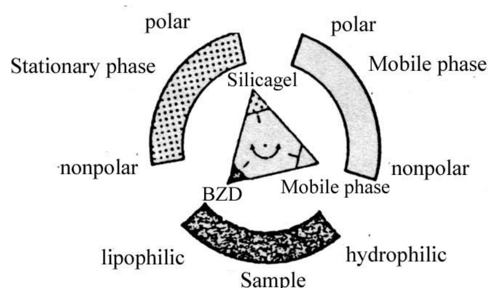

Fig. 2 *Stahl's triangle.*

After choosing the correct solvents, the next step was to adjust the solvent composition, in order to improve separation. As with selectivity, each solvent has its own polarity and consequently each solvent mixture has its own solvent strength. Calculation of a solvent mixture's strength is useful for comparison to other solvent mixtures, so solvent mixtures with the same strength but different selectivity can then be evaluated. The solvent strength of the mobile phases was calculated taking in consideration volume fractions and individual strength of each solvent from the mixture using the following formula [9]:

Mixture solvent strength = (solvent A % x solvent A strength)/100 + (solvent B % x solvent B strength)/100 + ….

Six mobile phases were selected and used (Table 1).

Table 1 Mobile phases selected for the separation of BZD derivates.

No.	Mobile phase	Solvent strength
I	ethyl acetate	0.58
II	chloroform – methanol 9:1	0.45
III	chloroform – acetone 4:1	0.52
IV	ethyl acetate – methanol - amoniac cc 17:2:1	0.63
V	hexan – chloroform –methanol 5:5:1	0.27
VI	acetone-chloroform-isopropanol 8:1:1	0.57

Fig. 3 and 4 show photographs of the chromatograms obtained with mobile phase IV in UV light at 254 and 366 nm respectively.

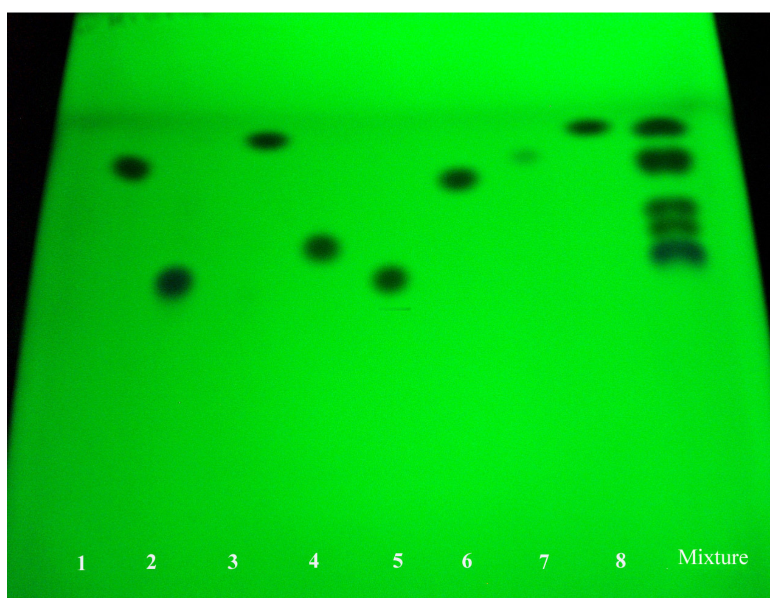

Fig. 3 *Chromatogram obtained using mobile phase IV (ethyl acetate – metahanol – conc. ammonia 17:2:1), detection in UV light at 254 nm (1 - nitrazepam, 2 - oxazepam, 3 - diazepam, 4 - chlordiazepoxide, 5 – alprazolam, 6 – bromazepam, 7 – chlorazepate potassium, 8 – medazepam).*

The Rf values, colours and fluorescence of the spots are mentioned in Table 2 and 3 respectively. The responses to UV radiation, those after exposure to sulphuric acid, and those to Dragendorff reagent were different. The colours and fluorescence of the spots are increased by treating BZDs with concentrated acids (concentrated sulphuric acid), because of formation of highly fluorescent derivates. We found that dipping the plate in concentrated acids increases the detection sensitivity of some BZDs, especially for chlordiazepoxide. The coloration conferred by Dragendorff reagent (solution of potassium bismuth iodide) was slightly less sensitive in some cases. Chlorazepate being used as a potassium salt was not detectable with Dragendorff reagent.

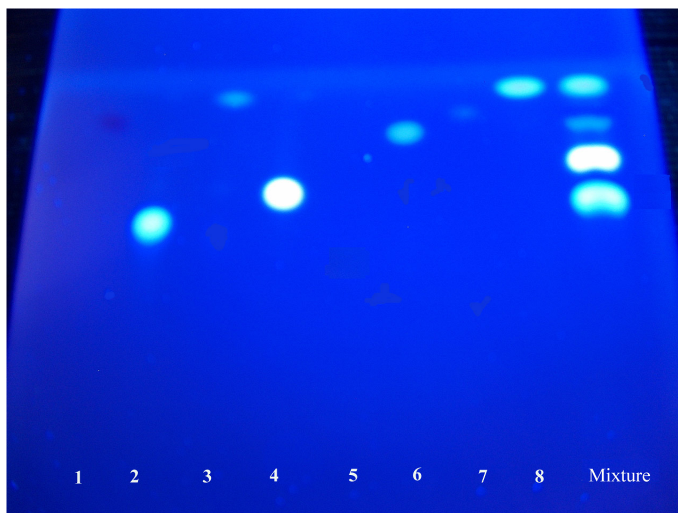

Fig. 4 *Chromatogram obtained using mobile phase IV (ethyl acetate – metahanol – conc. ammonia 17:2:1), detection in UV light at 366 nm (1 - nitrazepam, 2 - oxazepam, 3 - diazepam, 4 - chlordiazepoxide, 5 – alprazolam, 6 – bromazepam, 7 – chlorazepate potassium, 8 – medazepam).*

Table 2 Rf values of BZD in the six development system.

Benzodiazepine	Rf values					
	I	II	III	IV	V	VI
Alprazolam	0.19	0.42	0.18	0.57	0.2	0.4
Bromazepam	0.42	0.48	0.22	0.77	0.25	0.74
Chlorazepate	0.77	0.65	0.46	0.84	0.39	0.87
Chlordiazepoxide	0.33	0.58	0.23	0.62	0.32	0.75
Diazepam	0.78	0.85	0.67	0.87	0.64	0.86
Medazepam	0.60	0.88	0.70	0.89	0.8	0.86
Nitrazepam	0.77	0.75	0.45	0.78	0.47	0.87
Oxazepam	0.68	0.55	0.32	0.58	0.31	0.82

Table 3 Colours and fluorescence of BZD developed by the six TLC systems.

Benzodiazepine	Detection in (with)		
	UV 254	UV 366 nm	Dragendorff reagent
Alprazolam	brown	blue without fluorescence	pale orange
Bromazepam	brown	pale blue with fluorescence	gray-blue
Chlorazepate	pale blue	green with fluorescence	-
Chlordiazepoxide	brown	pale blue with fluorescence	pale orange
Diazepam	brown	green with fluorescence	pale orange
Medazepam	brown	pale green with fluorescence	orange
Nitrazepam	brown	blue without fluorescence	pale orange
Oxazepam	dark blue	pale blue with fluorescence	gray-blue

The stationary phase silicagel has surface Si-OH groups capable of forming hydrogen bonds with one another or with polar substances. A slight filtering action attributable to the pore structure of the silicagel can also affect separation.

Alprazolam has the lowest Rf values in all the studied systems because of its big tricyclic structure and high molecular mass. Oxazepam because of its polarity (the presence of a -OH group) exhibit lower Rf values than the structurally related diazepam or medazepam as it is more strongly bonded to the stationary phase. It is interesting also to notice that the N-substituted BZD (diazepam, medazepam) show in general the highest Rf values.

Hydrolysis in acid medium:

BZDs are relatively instable substance, because those easily hydrolyze in acidic solution and also decompose in UV light. Hydrolysis in acidic solution leads generally to 2-aminobenzophenone derivates, through the split of the N_1-C_2 bond of the diazepinic ring (Fig. 5) [10].

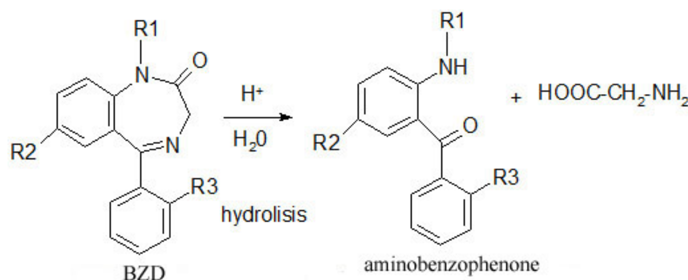

Fig. 5 *BZD degradation in acid medium.*

TLC of benzophenones obtained by acid hydrolysis of BZD derivates is widely used for identification purposes, but this method is not specific, as different BZD can give the same benzophenone derivate and others (alprazolam) do not form benzophenones. The advantage of using benzophenones TLC instead of the BZD themselves is that different metabolites of the same BZD can give the same benzophenone after hydrolysis, which makes this method more suitable for identification of these products from biological fluids [4].

To obtain a good hydrolysis it is necessary to cover the spots with a glass plate after moistening with sulphuric acid; omitting this detail causes probably rapid evaporation of sample with partial or no hydrolysis as a consequence. The use of hydrochloric acid instead of sulphuric acid is unsuitable when UV light is used for detection; background effects preventing the normal detection of spots [4].

Medazepam in acidic conditions exhibit a red spot on the plate, color that disappears when ammonia is sprayed on the spot. Alprazolam being a tryciclic BZD do not hydrolyze on the plate, and cannot be identified by the benzophenone method.

The primary aminobenzophenones obtained after hydrolysis (in the case of bromazepam, chlorazepate, chlordiazepoxide, oxazepam) can be identified on the chromatoplate by diazotation and with Bratton-Marshall reagent. After acid hydrolysis the plates were first sprayed with freshly prepared with a 1% aqueous sodium nitrite solution and oversprayed with Bratton Marshall reagent (N-(1-naphthyl)ethylendiamine in ethanol) (Fig. 6).

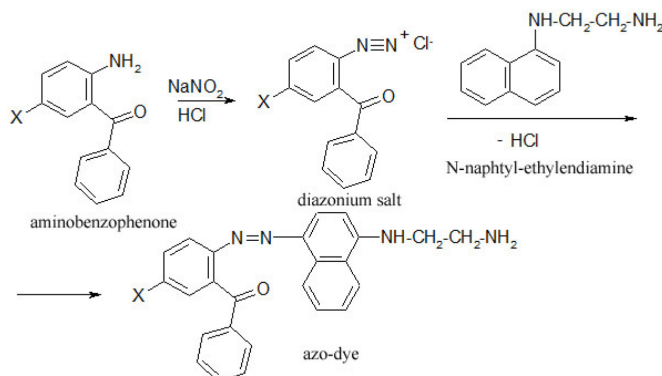

Fig. 6 *Bratton-Marshall reaction for primary aminobenzophenones.*

The reaction can also be executed by placing the plate in an empty chromatographic chamber, on the bottom of which a small beaker containing 20% aqueous sodium nitrite solution is placed, pipette a 25% hydrochloric acid into the beaker as fast as possible, to liberate nitrogen oxides, and seal the tank with its lid, for the diazotation of the aromatic primary amine groups. Finally the plate was sprayed with Bratton Marshall reagent to couple the diazonium salts. The resulting azo-dyes have a distinctive violet color. This procedure is very selective for BZD that form primary aminobenzophenones.

The plates clearly show that in addition to benzophenones, a considerable number of other hydrolysis products are formed. For bromazepam, chlordiazepoxide, diazepam, nitrazepam and oxazepam more than one hydrolysis products is noticeable on the plate.

4. Conclusions

We consider that it is convenient to check the identity of a BZD or of a mixture of BZD by the use of two or three chromatographic systems. This method is fast and reliable as three or even more system can run simultaneously at the same time and many samples can be put on the same plate.

Separation of BZD using TLC can be solved using the intact molecules or the benzophenones obtained after in situ acid hydrolysis, and is proving to be a very useful method in the preliminary analysis of these derivates.

The chromatographic systems presented in this paper permit an easy and rapid identification of a wide range of BZD currently in use.

REFERENCES

1. Cristea, A. (2006) **Tratat de farmacologie**, Editura Medicală, Bucureşti, 54-65.
2. Wall, P. (2005) **Thin-layer chromatography – A modern practical approach**, RSC Chromatography Monographs, Royal Society of Chemistry, London.
3. *** (2010**) European Pharmacopoeia**, 7th edition, Council of Europe, Strasbourg.

4. Schütz, H. (1996) **Dünnschichtchromatograpische Suchanalyse für 1,4-Benzodiazepine in Harn, Blut und Mageninhalt. Mitteilung VI der Denatskommision für Klinisch-toxikologische Analytik**, VCH Verlagsgessellschaft mbH, Stuttgart, 101-116.

5. Bakavoli, M., Kaykhaii, M. (2003) *Journal of Pharmaceutical and Biomedical Analysis* **31**, 1185-1189.

6. Otsubo, K., Seto, H., Futagami, K., Oishi, R. (1995) *Journal of Chromatograhy B* **669**, 408-421.

7. Pachaly, P. (2010) **DC-Atlas – Dünnschicht Chromatographie in de Apotheke**, Ed. Wissenschaftliche mbH, Stuttgart.

8. Fried, B., Sherma, J. (1999) **Thin layer chromatography**, 4th edition, Marcel Dekker Inc, New York.

9. Cimpoiu, C., Hodişan, T. (1999) *Journal of Pharmaceutical and Biomedical Analysis* **21**, 895-900.

10. Palage. M., Mureşan, A. (2006) **Medicaţia afecţiunilor sistemului nervos central**, Editura Medicală Universitară "Iuliu Haţeganu", Cluj-Napoca, 54-78.