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Review

Analysis of the 1,4-benzodiazepines by methods based on hydrolysis

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Abstract: The analytical applications of the hydrolysis of 1,4-benzodiazepines to the corresponding benzophenone derivatives are reviewed according to the analytical methods used for their final determination. The scope and limitations of the individual methods for the hydrolysis products are discussed: colour reactions, photometry, fluorimetry, thin-layer chromatography, gas chromatography and high-performance liquid chromatography. Published data on the kinetic parameters and mechanisms are summarized in an attempt to explain the problems involved in exploiting this reaction for analytical applications.

Keywords: *1,4-Benzodiazepines; 1,4-benzodiazepine hydrolysis; TLC of benzophenone derivatives; gas–liquid chromatography of benzophenone derivatives; high-performance liquid chromatography of benzophenone derivatives; fluorimetry of 1,4-benzodiazepines.*

Introduction

Since their introduction in the early 1960s, 1,4-benzodiazepines have made a substantial therapeutic contribution matched by few other drugs. As a consequence of their rapid development, the number of published analytical methods for their detection, identification and determination is large and, indeed, still increasing, especially for bioanalytical studies. Most of these analytical methods have been based on the physical and chemical properties of the original compounds, as discussed in two pertinent reviews [1, 2]. It has been found, however, that colour reactions of the original 1,4-benzodiazepines are not sufficiently sensitive and that the thermal stability of some compounds is questionable. Therefore many authors have proposed the conversion of 1,4-benzodiazepines to yield products which can be more easily evaluated by individual analytical methods.

Hydrolysis in acidic media has been found to be a reaction yielding products with

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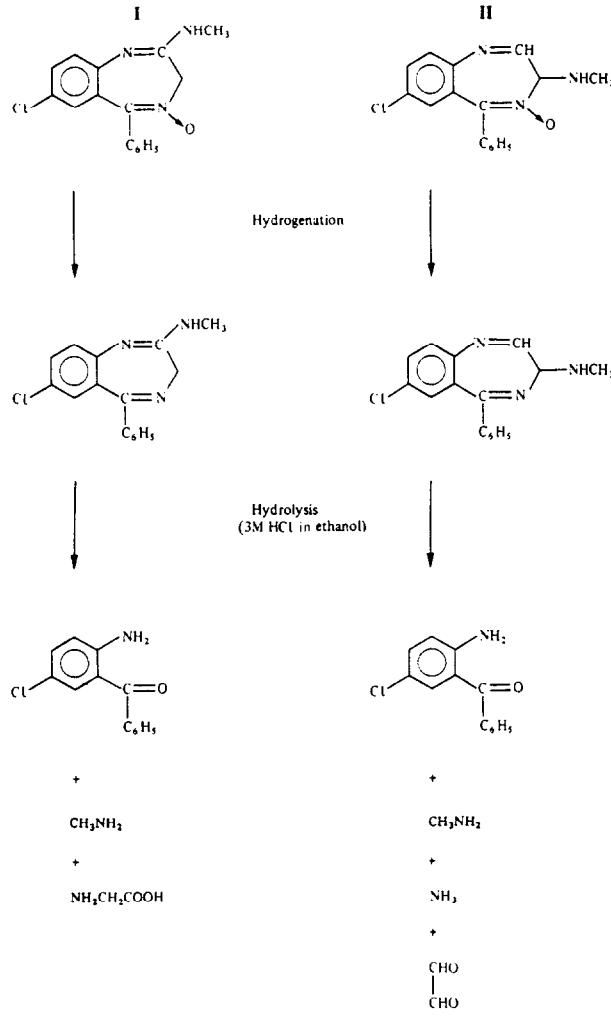
suitable analytical properties. It was used for the first time by Sternbach *et al.* [3, 4] in order to establish which of the structures **I** and **II** (Scheme 1) correctly described the first compound introduced in pharmacotherapy under the name Librium. 2-Amino-5-chlorobenzophenone, glycine and methylamine were found in the reaction mixture after boiling the hydrogenated product with ethanolic hydrochloric acid for 19 h, thus confirming structure **I** (Scheme 1). The reaction was also used for other 1,4-benzodiazepines to establish their structures [5].



Scheme 1

During the subsequent 20 years, this reaction has been used by many analysts who have primarily exploited the aminobenzophenones formed. Other products of hydrolysis, however, have been measured analytically in some cases.

Analytical Applications

Analytical procedures for 1,4-benzodiazepines based on hydrolysis are summarized in this section and are dealt with according to the analytical methods used for the final determination of the hydrolysis products, as indicated in Table 1.

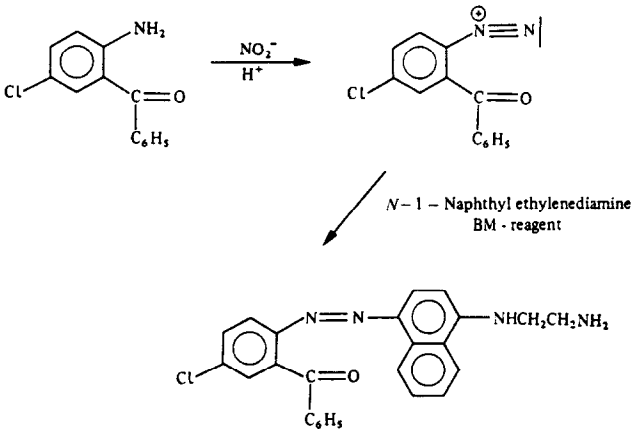
Table 1
Analytical methods based on the acid hydrolysis of 1,4-benzodiazepines

Analytical method used for determination of the hydrolysis products	References
Colour reactions	6–13
UV-visible spectrophotometry	14–41
Fluorimetry	16, 42–61
Paper and thin-layer chromatography	49, 50, 62–98
Detection on thin-layer chromatograms	88, 99–117
High-performance liquid chromatography	116–118
Gas chromatography	92, 119–152
Other methods	154–155

The benzophenones formed by the different 1,4-benzodiazepines commonly used in pharmacotherapy and by their metabolites are illustrated in Table 2. The breakdown of different 1,4-benzodiazepines to the same benzophenone would seem to be a certain disadvantage of the method. On the other hand, the 1,4-benzodiazepines and their metabolites, present in body fluids in both the free and conjugated forms, are all converted to the same benzophenone, thus yielding increased sensitivity, which can be important for example in clinical toxicology. Two or three different benzophenones can be formed in some cases, as is apparent for example, in the cases of nitrazepam, flunitrazepam and clonazepam.

Detection and identification by colour reactions

The primary aromatic amino group in benzophenones obtained by hydrolysis is usually exploited to achieve sensitive colour reactions for detection and identity tests of those 1,4-benzodiazepines where the N_1 atom is not substituted. Diazotization and coupling with 2-naphthol [6], *N*-(1-naphthyl)-*N'*, *N'*-diethylpropylenediamine [10], or *N*-(1-naphthyl)ethylenediamine (Bratton–Marshall reagent) [7, 8, 11–13] lead to the corresponding azo dyes (Scheme 2). In particular, the protonated species of the dye



Scheme 2

Table 2
Benzophenones formed from individual 1,4-benzodiazepines and their metabolites [118]

1,4-Benzodiazepine	Urinary metabolites	Corresponding benzophenone(s)
Bromazepam	3-Hydroxybromazepam	2-(2-Amino-5-bromobenzoyl)pyridine
	2-(2-Amino-5-bromobenzoyl)pyridine	
	2-(2-Amino-5-bromo-3-hydroxy-benzoyl)pyridine	
Camazepam	*N*-Methyloxazepam	2-Methylamino-5-chlorobenzophenone
Chlordiazepoxide	Demethylchlordiazepoxide	2-Amino-5-chlorobenzophenone
	Demoxepam	
	Nordiazepam	
Clonazepam	7-Aminoclonazepam	2-Amino-5-nitro-2'-chlorobenzophenone
	7-Acetamidoclonazepam	2,5-Diamino-2'-chlorobenzophenone
	3-Hydroxyclonazepam	
	7-Amino-3-hydroxyclonazepam	
Di-potassium clorazepate	Nordiazepam	2-Amino-5-chlorobenzophenone
	Oxazepam	
Diazepam	Temazepam	2-Methylamino-5-chlorobenzophenone
	Nordiazepam	2-Amino-5-chlorobenzophenone
	Oxazepam	
Flunitrazepam	Norflunitrazepam	2-Amino-5-nitro-2'-fluorobenzophenone
	7-Aminoflunitrazepam	2-Methylamino-5-nitro-2'-fluoro-benzophenone
	3-Hydroxyflunitrazepam	2-Methylamino-5-amino-2'-fluoro-benzophenone
Lorazepam	Almost not metabolized but excreted as glucuronide	2-Amino-5,2'-dichlorobenzophenone
Medazepam	Oxazepam	2-Methylamino-5-chlorobenzophenone
	Diazepam	2-Amino-5-chlorobenzophenone
	Normedazepam	
	Nordiazepam	
	Dehydromedazepam	
Nitrazepam	2-Amino-5-nitrobenzophenone	2-Amino-5-nitrobenzophenone
	2-Amino-3-hydroxy-5-nitro-benzophenone	2,5-Diaminobenzophenone
	7-Aminonitrazepam	
	7-Acetamidonitrazepam	
Oxazepam	Not metabolized but excreted as glucuronide	2-Amino-5-chlorobenzophenone
Prazepam	Nordiazepam	2-Cyclopropylamino-5-chlorobenzophenone
	Oxazepam	
Temazepam	Oxazepam	2-Methylamino-5-chlorobenzophenone
		2-Amino-5-chlorobenzophenone

produced by BM-reagent is intensely coloured (Scheme 2). This reaction has been involved in the official identity tests for oxazepam and for chlordiazepoxide, as described in the *Pharmacopoeias* [11–13]. The reaction has also been used for detection in thin-layer chromatography.

Spectrophotometry

No suitable colour reactions are available for 2-methylamino-5-chlorobenzophenone formed by the hydrolysis of diazepam. The compound itself, however, is intensely yellow in colour and can therefore be determined at 410 nm directly [34]. Sensitive colour reactions are available for benzophenones containing primary aromatic amino groups, as summarized in Table 3.

Table 3
Procedures used for the photometric determination of 1,4-benzodiazepines after hydrolysis

1,4-Benzodiazepine	Reagent	References
Chlordiazepoxide	1,2-Naphthoquinone-4-sulfonate	27
	Diazotization and coupling with BM-reagent*	14, 15, 17, 21, 25, 32, 37, 39
	Diazotization and coupling with 1-naphthylamine	19
	Diazotization and coupling with thiocol	23
	Diazotization and coupling with 1-naphthol	26
	Diazotization and coupling with 8-hydroxyquinoline	36
	Diazotization and coupling with thymol	35
Oxazepam	1,2-naphthoquinone-4-sulfonate	30
	Diazotization and coupling with BM-reagent*	16, 21
	Diazotization and coupling with 1-naphthol	22, 26
	Diazotization and coupling with thiocol	23
	Diazotization and coupling with 8-hydroxyquinoline	38
Nitrazepam	1,2-Naphthoquinone-4-sulfonate	30
	Diazotization and coupling with BM-reagent*	21, 24, 39
	Diazotization and coupling with 8-hydroxyquinoline	38
	Diazotization and coupling with 1-naphthol	26
	Diazotization and coupling with thiocol	23
Bromazepam	Diazotization and coupling with BM-reagent*	28
Dipotassium clorazepate	Diazotization and coupling with BM-reagent*	20
Clonazepam	1,2-Naphthoquinone-4-sulfonate	40

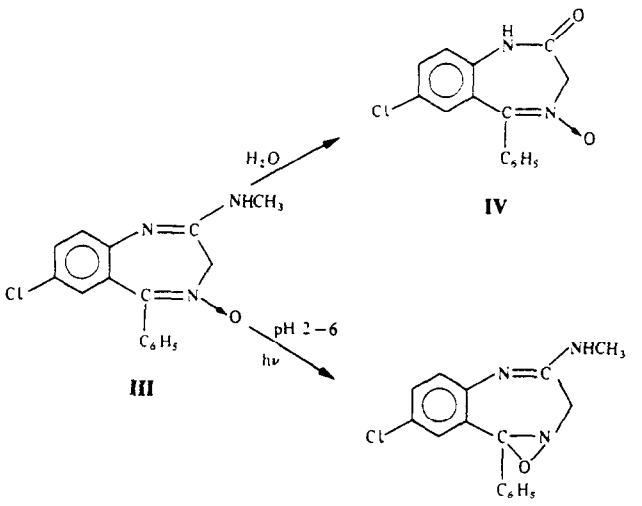
* BM-reagent = Bratton–Marshall Reagent, *N*-(1-naphthyl)ethylenediamine.

Fluorimetry

Most of the 1,4-benzodiazepines exhibit fluorescence in acid solutions in the Hammett acidity region. It has been shown that the aryl azomethine function is a prerequisite for fluorescence [54]. The structure of the molecule being excited also depends on the acid–base equilibria occurring in the ground state [54]. Further fluorescent species can be produced photometrically, thermally in acidic solutions and also synthetically, by derivatization of the products of acid hydrolysis.

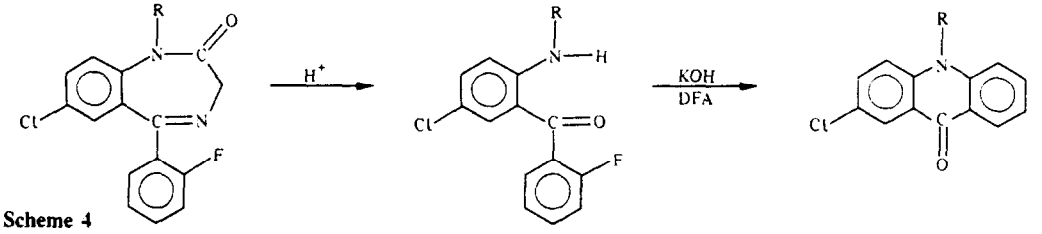
Thus, mild hydrolysis of chlordiazepoxide (**III**, Scheme 3) yields the lactam (**IV**). Photolytic rearrangement in alkaline medium gives compound **V**, containing a 4,5-epoxide group [155–157] and exhibiting intense fluorescence [42, 43, 58]. This reaction is specific for 1,4-benzodiazepines containing the N→O group.

Scheme 3



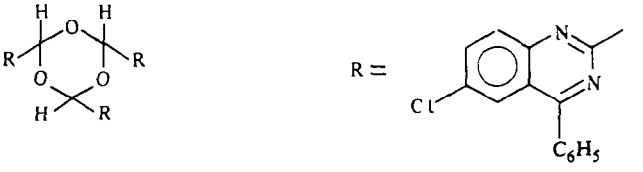
III IV V

1,4-benzodiazepines containing an ortho-halogen substituent in the 5-phenyl ring are hydrolysed to the corresponding benzophenones, which are converted to highly fluorescent acridanones [49, 50, 55, 56] by the reaction with KOH in dimethylformamide (Scheme 4).

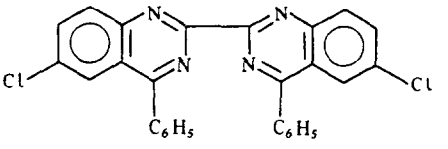


Scheme 4

Oxazepam exhibits a very intense fluorescence in alcoholic phosphoric acid solution after mild heating [44, 45]. The fluorescence was originally ascribed [51] to the formation of a trimer of the 6-chloro-5-phenylquinazoline-2-carboxyldehyde (**VI**). The compound



VI

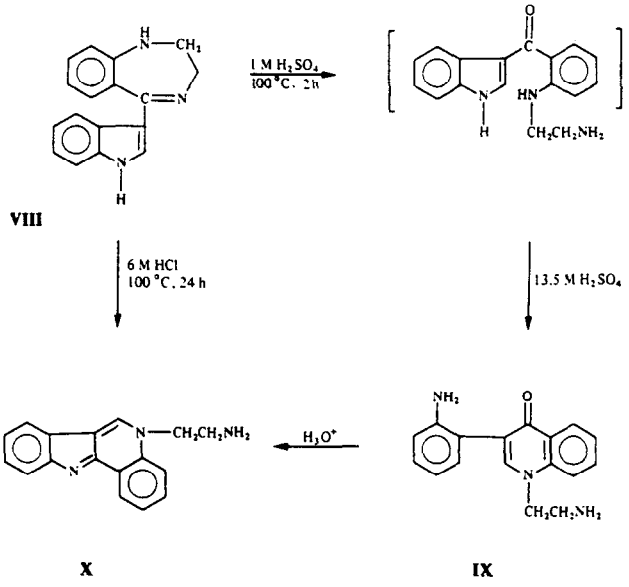


VII

actually isolated (melting point 316°C) from the reaction mixture by the Canadian authors was, however, shown not to be the fluorescing species, but a dimeric molecule of structural formula **VII**, formed on pouring the reaction mixture into water [158].

A highly specific and sensitive assay [53] for the 5-indolyl-1,4-benzodiazepine **VIII** is based on its rearrangement in concentrated sulphuric acid at elevated temperatures, yielding a phenylquinolone (**IX**) which fluoresces strongly in concentrated sulphuric acid. Prolonged hydrolysis of **VIII** leads to the formation of the indoloquinoline (**X**), which is also highly fluorescent but at different emission and excitation wavelengths (Scheme 5).

Scheme 5



The products of 1,4-benzodiazepine hydrolysis can be determined fluorimetrically by reacting with some of the commonly used fluorescent labelling reagents. Thus, the reaction with *o*-phthalaldehyde [52, 61] can be used for the hydrolysis products of nitrazepam and its metabolites and similarly, methylamine produced by hydrolysis of chlordiazepoxide can be labelled with fluorescamine [57].

Further methods for the fluorimetric determination of some common 1,4-benzodiazepines are summarized in Table 4.

Table 4
Fluorimetric determination of individual 1,4-benzodiazepines

1,4-Benzodiazepine	References
Chlordiazepoxide	42, 43, 48, 55, 57, 59, 61
Diazepam	55
Nitrazepam	52, 60, 61
Oxazepam	16, 44–46, 48, 51, 55, 61
Medazepam	47
Tetrazepam	49
Flurazepam	50
Chlorazepate	55, 61
Demoxepam	58, 61
Lorazepam	61

Thin-layer chromatography (TLC)

The four approaches to exploiting the hydrolytic reaction in TLC are summarized below.

TLC of original 1,4-benzodiazepines, followed by detection after direct hydrolysis on the plate. The original compounds can be separated by TLC using similar conditions as for other synthetic drugs of basic character [1, 159]. Quantitation can be achieved by densitometry *in situ* or by spectrophotometry after elution from the plate. A major problem is the lack of sensitive methods for detection or densitometric measurement. It is therefore advantageous to use a hydrolytic reaction where the developed and dried chromatogram is sprayed with hydrochloric or sulphuric acid solution, heated at 110–130°C for the time required to convert the original compounds to the respective benzophenones, and the primary aromatic amino group diazotized in an atmosphere of nitrous acid fumes, with coupling to *N*-(1-naphthyl)ethylenediamine reagent (cf. Scheme 2). Secondary aminobenzophenones which do not react can be dealkylated prior to detection, to give the primary aminobenzophenones, using photolysis directly on the plate [115]. Another possibility is to convert the 1,4-benzodiazepines after separation, to yield fluorescent spots by the action of mineral acids (HCl, H_3PO_4, H_2SO_4) directly on the plate, followed by spectrofluorodensitometric measurement [46, 59, 102, 103, 106–108].

TLC of the corresponding benzophenones. In the same way as for other groups of synthetic drugs, 1,4-benzodiazepines can be readily measured analytically after acid hydrolysis. This procedure is widely used as a screening technique for the analysis of body fluids. 1,4-Benzodiazepines can be present in body fluids as original compounds, their metabolites and conjugates, all of which generally yield the same benzophenone, thereby increasing the sensitivity of the analytical procedure. The aminobenzophenones which are formed by hydrolysis prior to TLC are detectable by diazotization of the primary aromatic amino group and coupling with *N*-(1-naphthyl)ethylenediamine (Scheme 2). Benzophenones containing a secondary amino group give no reaction. They can, however, be photolytically dealkylated directly on the plate prior to diazotization, as discussed above [115]. One advantage of separating the benzophenones instead of the original compounds is that good separation efficiency can be obtained in simple solvent systems, together with good stability of the species chromatographed and high detection sensitivity. References to individual benzodiazepines chromatographed after hydrolysis are summarized in Table 5.

TLC of acridanones. Conversion of the original 1,4-benzodiazepines to the corresponding 9-acridanones (Scheme 4) prior to TLC can provide high sensitivity for spectrofluorodensitometric determination, specificity being ensured by the TLC separation itself [49, 50, 84, 85].

Two-dimensional TLC with hydrolysis to the benzophenone. In this procedure the original 1,4-benzodiazepines are separated in the normal direction by thin-layer chromatography, followed by hydrolysis to the corresponding benzophenones directly on the plate; the benzophenones are then separated by chromatography in the second direction, followed by detection by diazotization and coupling [111–115]. This technique, often referred to as the 'Trennung–Reaktion–Trennung' (TRT) technique, is

Table 5
TLC of the hydrolysis products of individual 1,4-benzodiazepines

1,4-Benzodiazepine	References
Diazepam	49, 63–66,70–72, 74–76, 78, 80, 81, 83, 87, 89, 93–95, 98
Flurazepam	50, 84, 86, 87, 90, 94, 95, 98
Nitrazepam	49, 65, 71, 72, 76, 81–83, 87, 90, 94, 95, 98
Clonazepam	86, 90, 94, 95, 98
Chlordiazepoxide	49, 65, 66, 70–77, 81, 83, 87, 89, 90, 95, 98
Bromazepam	90, 94, 98
Lorazepam	76, 79, 83, 87, 90, 94, 98
Oxazepam	49, 67, 70–72, 76, 81, 82, 87, 90, 94, 98
Flunitrazepam	90, 94, 95, 98
Tetrazepam	49, 71
Di-potassium clorazepate	49, 70, 71, 76, 87, 90, 94, 98
Temazepam	90, 94
Camazepam	91, 94
Prazepam	68–70, 83, 87, 90, 94, 97, 98
Tetrazepam	85

suitable in cases when 1,4-benzodiazepines can not be resolved as the original compounds by TLC, but can be resolved as the benzophenones.

High-performance liquid chromatography (HPLC)

HPLC is a versatile technique that has been used to advantage with the availability of highly sensitive spectrophotometric, fluorimetric and electrochemical detectors. The aromaticity of the benzodiazepines renders them suitable for UV spectrometric detection, while those which can be derivatized to yield either a quinazolinone (demoxepam) or acridanone (flurazepam) derivative can be readily determined by fluorescence. HPLC lends itself to the separation of the major metabolites in urine and also to the analysis of thermally unstable compounds, such as chlordiazepoxide, oxazepam and lorazepam.

HPLC of the hydrolysis products of 1,4-benzodiazepines has been preferred for detecting benzodiazepines in clinical emergencies. Chromatography of the benzophenones leads to a loss of specificity, but to a gain in sensitivity, since the benzodiazepines themselves and their metabolites, free or conjugated, yield the same benzophenone or benzophenones. This potential decrease in specificity should be offset against the possibility of screening all of these benzodiazepines simultaneously in a reasonable time [116–118, 160].

Gas–liquid chromatography (GLC)

The majority of GLC assays reported [1, 159, 160] for the determination of individual benzodiazepines in biological fluids use electron capture detection due to the presence of an electronegative group, usually a halogen or nitro group, in the 7-position of the molecule. A halogen in the 2′-position of the 5-phenyl ring and a carbonyl group in the 2-position of the 1,4-benzodiazepine ring can also contribute to this detector response. Conversion of the 1,4-benzodiazepines to the corresponding benzophenones is advantageous in situations where the analysis of the intact compound is not feasible, due either to poor intrinsic sensitivity by electron-capture detection or to thermal instability. For example, conversion of diazepam to 2-amino-5-chlorobenzophenone results in a ten-fold

increase in sensitivity by comparison with detection of the parent compound [119]. Although a major criticism of this procedure is the lack of specificity, since more than one compound can yield the same benzophenone, it is still valid where the parent drug is the only component in blood to yield a specific benzophenone as, for example, with clonazepam, oxazepam or lorazepam.

The 3-hydroxyl-1,4-benzodiazepinones such as oxazepam and lorazepam undergo thermolytic rearrangement to yield their respective quinazoline-carboxaldehydes [161–165] which can result in poor precision and reproducibility. Although these compounds can be analysed as their quinazoline-carboxaldehydes by direct injection [116–171], they can be analysed with higher sensitivity and better reproducibility by acid hydrolysis to their benzophenones [133, 134, 160].

Examples of GLC analysis of the hydrolysis products of individual 1,4-benzodiazepines are given in Table 6.

Table 6
GLC of the hydrolysis products of individual 1,4-benzodiazepines

1,4-Benzodiazepine	References
Diazepam	47, 119–127, 130, 142, 148, 150–152
Flurazepam	150–152
Nitrazepam	127, 129, 130, 132, 139, 144, 150–152
Clonazepam	132, 136–138, 145, 149–152
Chlordiazepoxide	121, 125, 127, 130, 151, 152
Bromazepam	121, 140, 151, 152
Lorazepam	128, 151, 152
Oxazepam	125, 127, 130, 133, 134, 151, 152
Flunitrazepam	136, 145, 151, 152
Tetrazepam	127, 151
Di-potassium clorazepate	130, 141, 143, 151, 152
Temazepam	131
Camazepam	151, 152
Prazepam	151, 152

Reaction Conditions

The reaction conditions necessary for successful application of hydrolysis to analytical procedures have usually been established experimentally. They therefore differ considerably from one author to another for individual compounds. Examples of reaction parameters given in the literature are given in Table 7. It should be emphasized that the poor solubility of both the original 1,4-benzodiazepines and their benzophenones does not lead to any difficulties when the reaction is carried out in aqueous media on the analytical scale, owing to the low concentrations of the analytes. On the other hand, whenever the corresponding benzophenones have been prepared by preparative hydrolytic reactions, organic solvents (most often alcohols) have had to be added to ensure a homogenous solution [3, 5, 129, 173–178].

The reactions used by analysts have usually been assumed to proceed quantitatively. Only a few authors have investigated the yield of benzophenones. Their results, summarized in Table 8, show that the yields differ considerably in the case of individual 1,4-benzodiazepines.

Table 7
Reaction conditions for the hydrolysis of 1,4-benzodiazepines used in analytical procedures

Acid used and its concentration	Time/Temperature	References
6 M H_2SO_4	1 h/100°C	128, 133
3 to 4 M H_2SO_4	7 to 10 min	134
3 M H_2SO_4	0.5 to 2 h/100°C	144
	1.5 h/100°C*	145
	2.5 h/100°C	121
1 M H_2SO_4	2 h/100°C	53
0.5 M H_2SO_4	2 h	137
6 M HCl + 3 M H_2SO_4 (95:5 v/v)	1 h/100°C	95, 136
conc. HCl–methanol–ether 1:3:1 v/v	10 min/reflux	149
24% HCl–ethanol		87, 40
conc. HCl	0.5 h/reflux	83
	12 h/100°C	63
	12 h/100°C	62
6 M HCl	0.5 h/125°C	7, 14, 15, 32
	1 h/60°C	52
	1 h/100°C	16, 64, 80, 84, 97, 119, 122–124, 132, 141, 142
	45 min/100°C	57
	30 min/100°C	74
5 to 6 M HCl	2 h/100°C	70
5 M HCl	1 h/100°C	68, 69
	0.5 h/100°C	154
4 M HCl	2 h/100°C	50
	0.5 h/100°C	10, 65, 71
3 M HCl	10 to 30 min/reflux	21, 24, 35, 129
	0.5 h/100°C	
2 to 3 M HCl	30 min/100°C	172
2.8 M HCl	2 h/100°C	19
	15 min/100°C	151
2.5 M HCl	0.5 h/reflux	66
2 M HCl	20 min/120°C	37
	2 h/100°C	22
1 M HCl	120°C	23
0.1 M HCl	15 min*	81

* Sealed glass ampoule.

Reaction Mechanisms

Much work has been carried out during the last ten years to elucidate the chemical changes taking place during the hydrolysis of individual 1,4-benzodiazepines. Various methods have been used to investigate the kinetics of hydrolysis and much care has been devoted to the isolation of intermediate products in order to elucidate the reaction mechanisms. It has been found that the reaction is complex and is influenced by many factors: for example, the functional groups present in the 1,4-benzodiazepine molecules,

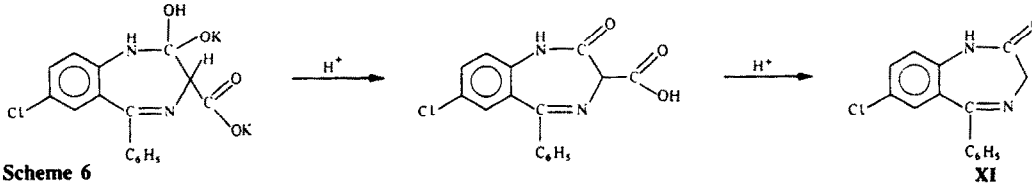
Table 8
Yields of hydrolytic products reported by different authors

1,4-Benzodiazepine	Conditions	Yield (%)	References
Chlordiazepoxide	10% HCl/15 min	96.9	21
	10% HCl/15 min	60.0	118
Oxazepam	10% HCl/15 min	85.0	21
	10% HCl/15 min	83.0	118
	3 to 4 M H_2SO_4/7 to 10 min	95.0	134
	6 M HCl	50.0	134
Nitrazepam	5 M HCl/2 h	95.0	178
	3 M H_2SO_4/30 min	90.0	129, 144
	10% HCl/15 min	98.0	21
		93.0	118
Bromazepam	10% HCl/15 min	42.0	118
Flunitrazepam	10% HCl/15 min	81.0	118
Desmethylflunitrazepam	10% HCl/15 min	93.0	118
Diazepam	10% HCl/15 min	74.0	118
Temazepam	10% HCl/15 min	99.0	118
Camazepam	10% HCl/15 min	99.0	118
Medazepam	10% HCl/15 min	0	118
Di-potassium clorazepate	10% HCl/15 min	30.0	118
N-Desmethyldiazepam	10% HCl/15 min	94.0	118
Lorazepam	10% HCl/15 min	65.0	118
Clonazepam	10% HCl/15 min	76.0	118
	37% HCl/5 min	95.0	179

their pK_a values, the type of acid used and its concentration, the presence of organic solvents used to achieve complete dissolution of the reactants, and so on. The reaction involves at least two steps, and side reactions resulting in the formation of by-products have been observed. Some basic rules characterizing the chemical changes commonly involved can be summarized as follows.

(1) The first reaction step observed under very mild conditions in aqueous solutions is the hydrolytic removal of some functional groups present in the original benzodiazepine molecule, the basic seven-membered structure remaining unchanged. Thus chlordiazepoxide or its protonated form are easily hydrolysed to their lactam, demoxepam, as shown in Scheme 3. The presence of the oxazirane derivative in the reaction mixture is due to the action of light (cf. Scheme 3).

Dipotassium clorazepate undergoes decarboxylation even under the mildest conditions, for example on a HPLC column, nordiazepam (*N*-desmethyldiazepam, **XI**) being formed [180, 181] (Scheme 6).

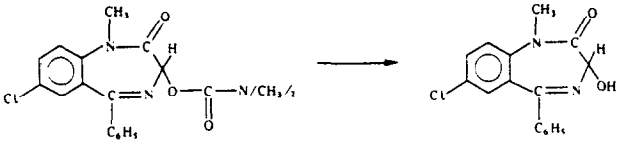


Scheme 6

XI

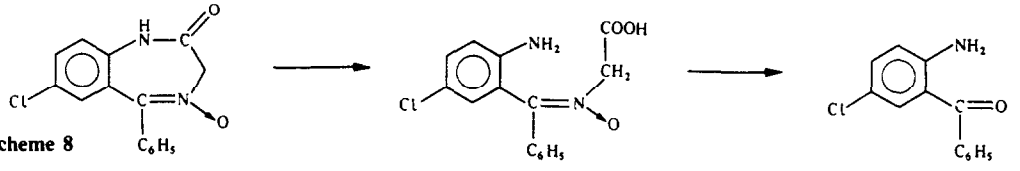
The decarboxylation process of dipotassium clorazepate has been shown to be acid-catalysed, the rate of the reaction increasing with decreasing pH of the solution [182]. The reaction is pseudo-first order [183]. Camazepam, the dimethylcarbamate ester of temazepam, is hydrolysed quantitatively to temazepam in 2 M HCl at 40°C within 5 h (Scheme 7) [184].

Scheme 7



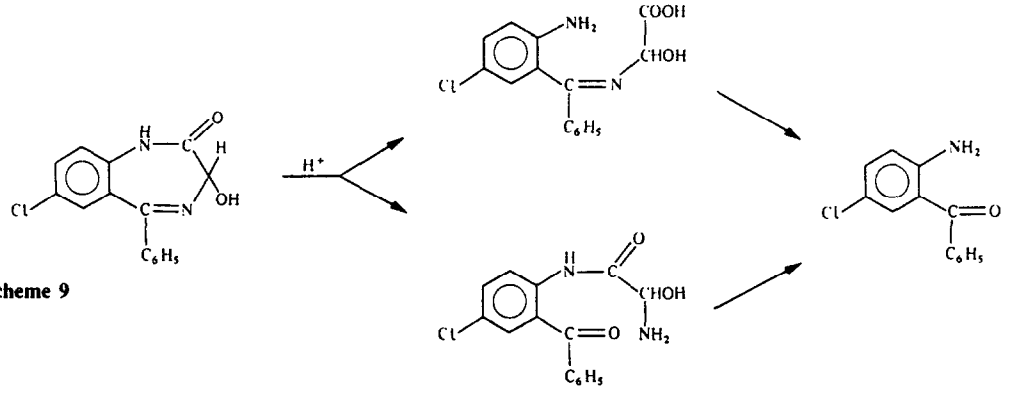
(2) The first reaction step in which the 1,4-benzodiazepine ring system is attacked represents two parallel reactions involving ring opening at the 1,2-amidic or 4,5-azomethine bonds. Amide bond cleavage seems to be preferred in the case of chlordiazepoxide or demoxepam (Scheme 8) [185, 186].

Scheme 8

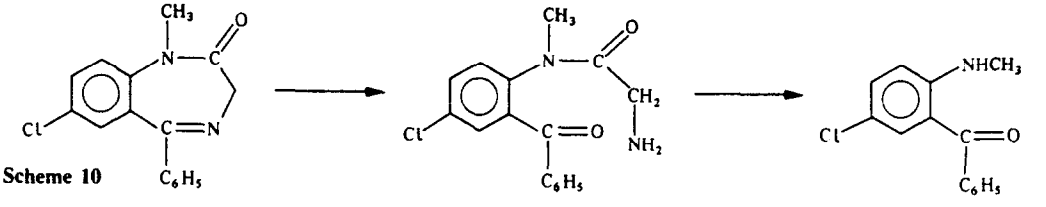


The formation of both intermediates, i.e. compounds formed by the cleavage of the 1,2-amidic bond as well as the 4,5-azomethine bond, has been observed in the case of oxazepam (Scheme 9). At pH values above the pK_a of oxazepam cleavage of the 1,2-amidic bond is preferred, whereas protonation of the azomethine nitrogen at lower pH favours azomethine bond cleavage [185, 187]. The hydrolysis of diazepam, nitrazepam, flurazepam, flunitrazepam, etc. has been found to proceed exclusively via the breakdown of the 4,5-azomethine bond (Scheme 10) [186–188].

Scheme 9



Scheme 10



The first-order rate constants and half-lives of the hydrolysis reaction of several 1,4-benzodiazepines in 1 M and 0.1 M HCl at 25°C are given in Table 9. It is evident that compounds hydrolyse more rapidly at lower acid concentrations [189–191].

Table 9
Kinetic data for some 1,4-benzodiazepines at 25°C [191]

1,4-Benzodiazepine	1 M HCl		0.1 M HCl	
	k (10^{-4} μA.s^{-1})	$t_{1/2}$ (min)	k (10^{-4} μA.s^{-1})	$t_{1/2}$ (min)
Ro 5-4781*	0.39	299	2.62	44
Ro 5-6728†	0.28	415	0.82	141
Di-potassium clorazepate	—	> 24 h	0.05	2402
Medazepam	‡	—	—	—
Diazepam	—	> 24 h	—	> 24 h
Desmethyldiazepam	—	> 24 h	—	> 24 h
Nitrazepam	0.17	672	0.18	652
Ro 5-4435	0.52	224	3.03	38
Clonazepam	0.11	1069	0.41	285
Flunitrazepam	0.49	237	4.66	25
Flurazepam	1.93	60	5.38	21

* 5-Phenyl analogue of flurazepam.
† 5-*o*-chlorophenyl analogue of flurazepam.
‡ No detectable reaction.

The values of the rate constants differ considerably with the presence of certain substituents. The changes in the rate constant values brought about by the alteration of the 1-substituent suggest some involvement of this group in the rate-determining step of hydrolysis. It has been proposed that bulky groups, such as those in flurazepam, could interact with the azomethine group. If the 4,5-double bond is the centre of attack in the hydrolysis reaction, these facts would confirm that steric effects play a role.

Comparison of the rate constant values for compounds with varying 5-*o*-phenyl substituents indicates that in both acid solutions the descending order of rate constants is F > H > Cl [190, 191]. The fluorine atom seems to withdraw electronic charge from the azomethine group, resulting in a positively-charged nitrogen, which would account for the lower pK_a value. The slow rate observed for the *o*-chloro-derivative presumably occurs as a result of interference by the large chlorine atom in the hydrolytic reactions that involve the azomethine group.

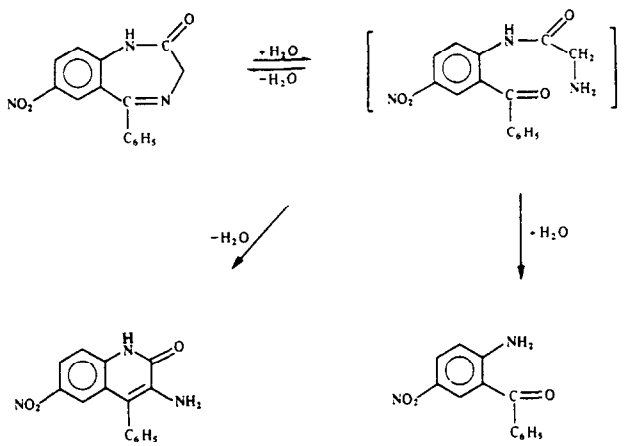
(3) The most important feature of the reaction step discussed above is its reversibility, especially at higher pH values. The intermediate of nitrazepam hydrolysis, for example, is rapidly cyclized to form nitrazepam at pH values above its pK_a value [190–195]. In analytical procedures the hydrolysis products are usually extracted into organic solvents after addition of alkali hydroxide to the acid reaction mixture. This could lead to formation of the original compound in certain cases. These properties of the intermediates must be taken into consideration whenever attempts are made to isolate these compounds.

(4) The second reaction step involves cleavage of the remaining 1,2-amidic or 4,5-azomethine group and the formation of the final hydrolysis products, the 2-amino-5-substituted benzophenones and glycine derivatives (cf. Schemes 8–10). This reaction step is not reversible and takes place under more drastic conditions (see Table 7). Some substituents on the 1,4-benzodiazepine skeleton can undergo corresponding chemical changes under these conditions, e.g. the trifluoromethyl group is hydrolysed to the carboxy group, which is cleaved [174]. Table 10 summarizes references to papers in which the hydrolysis of individual benzodiazepines has been studied kinetically.

Table 10
Kinetic studies published for individual 1,4-benzodiazepines

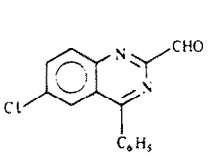
1,4-Benzodiazepine	References
Chlordiazepoxide	185, 186, 190, 195, 196
Diazepam	187, 190, 195, 197, 198, 205
Bromazepam	189, 190, 195, 199
Nitrazepam	188, 190, 193, 195, 198
Flunitrazepam	190, 195
Clonazepam	190, 195, 200
Demoxepam	186
Oxazepam	185, 187, 190, 205
Nimetazepam	193
Flurazepam	190, 191, 201
Nordiazepam	200
Di-potassium clorazepate	182, 183, 190
Medazepam	190
Prazepam	190
Lorazepam	190

(5) Several by-products have been isolated and identified, indicating that some side-reactions can take place. Contraction to a six-membered ring yielding the corresponding quinolone or quinazolone derivatives, respectively, is the most important side-reaction. Thus the by-product of nitrazepam hydrolysis is 3-amino-1,2-dihydro-6-nitro-4-phenyl-quinol-2-one (carbostyril), probably formed by cyclization of the glycylaminobenzophenone intermediate [198] (Scheme 11).

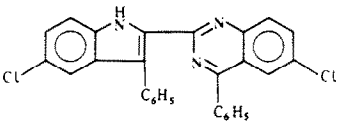


Scheme 11

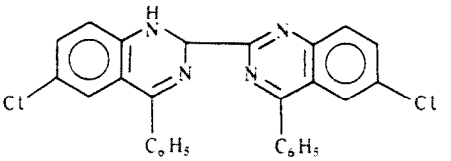
The hydrolysis of oxazepam is significantly dependent on the reaction conditions employed, and especially on the presence of organic solvents and the acid used. The formation of benzophenone is preferred in aqueous media, especially in sulphuric acid (see Table 8), whereas 6-chloro-4-phenylquinazolone-2-carboxaldehyde (**XII**) seems to be the first reaction step involving the contraction of the benzodiazepine ring when the reaction is carried out in the presence of organic solvents or in glacial acetic acid [158, 202–205]. This compound, however, easily undergoes further tranformation yielding dimeric products [51, 158], the structure of which (**VII, XIII**) has been determined in the authors' laboratory. Compound **VII** has also been isolated by Argentinian authors [206], who described it as structure **XIV**. The by-product **XIII** isolated in the present authors'



XII



XIII



XIV

laboratory [158] indicates further ring contraction to the five-membered ring. The formation of five-membered ring compounds from benzodiazepines is well known, but is more commonly observed in alkaline media [207].

The yields of compounds **VII** and **XIII** after acid hydrolysis of oxazepam were in the range of 45–50% when the reaction was carried out in hydrochloric acid–ethanol (1:9 v/v) mixtures [158, 205]. Table 11 gives the yields of 2-amino-5-chlorobenzophenone after the hydrolysis of oxazepam in mixtures of hydrochloric acid with different amounts of alcohols. A similar influence of the presence of organic solvents on the yields of the corresponding benzophenone has also been observed in the case of chlordiazepoxide [204].

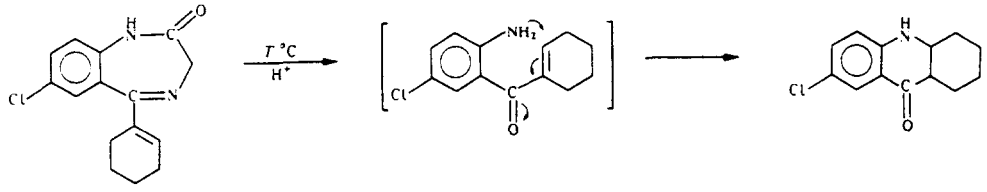
Table 11
Yields of 2-amino-5-chlorobenzophenone after acid hydrolysis of oxazepam, with respect to the type and concentration of alcohol [204]

Concentration of HCl	Concentration of alcohol (% v/v)	Yield (%)		
		Methanol	Ethanol	1-Propanol
HCl (conc.)	10	79	77	74
	30	69	60	59
	50	59	52	48
	70	53	47	42
	90	41	38	37
17.5% v/v HCl	10	70	67	61
	30	58	52	43
	50	49	40	36
	70	44	36	32
	90	36	28	24

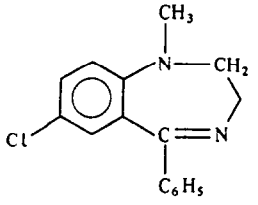
(6) Rearrangements of the 1,4-benzodiazepines also involve the 5-substituent in some cases. The corresponding acridanones can be formed, as shown in Scheme 4, in the case of compounds with an *o*-halogen substituent or a 1,2-double bond in the cyclohexene ring [49, 208] (cf. Scheme 12). The cyclization of 2-amino-2′-fluorobenzophenones to the corresponding acridanones is a common reaction [209]. A similar rearrangement is given in Scheme 5.

(7) Some problems seem to have been encountered in the case of compounds such as medazepam [**XV**], which do not possess the 1,2-amidic bond [49, 95, 112, 120, 126, 130, 210–212]. These compounds were probably hydrolysed at the 4,5-azomethine bond. However, the intermediates formed seem to be sensitive to acid–base conditions in the reaction mixture and easily undergo recyclization according to section 3 above.

(8) Another type of by-product is formed by the migration of halogen atoms, as observed in the case of clonazepam [136] and bromazepam [213]. 4-Bromo-2-(pyrid-2-

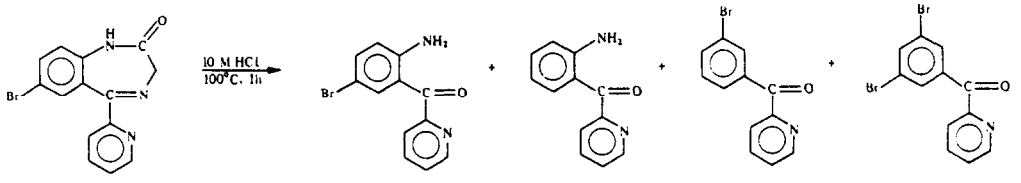


Scheme 12



XV

ylcarbonyl)aniline was the expected hydrolysis product of bromazepam and was confirmed when sulphuric acid was used. In hydrochloric acid, however, the formation of the 4-isomer and both the dehalogenated compound and the 2,4-dibromoderivative have also been observed (Scheme 13).



Scheme 13

Conclusions

The hydrolytic reaction of 1,4-benzodiazepines has been found to have wide application in the analytical chemistry of this group of compounds. Since the earliest analytical method based on this approach, much knowledge has been gained on the course of the reaction, its mechanism and kinetics, as well as on the formation of by-products. Knowledge of the necessary reaction parameters should enable further successful applications and will be helpful in the interpretation of results obtained in more complicated cases, including mixtures with metabolites. Such work will also enable the choice of reaction conditions to be made on a systematic basis for each particular benzodiazepine derivative.

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