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Review

Chromatographic separation of 2,3-benzodiazepines

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Abstract

A review of chromatographic methods for the determination of 2,3-benzodiazepines (2,3-BZs) is presented. The determinations are performed to investigate the presence of potential impurities in drug substances and to study their pharmacokinetic profile in biological samples, either in animals or in humans. Several methods dealt with a pretreatment of samples, i.e., liquid–liquid extraction by using a variety of solvents, solid-phase extraction, direct injection of specimens into the chromatographic apparatus. Different chromatographic techniques have been used. High-performance liquid chromatography allows optimal sensitivity and specificity by using ultraviolet or diode array detection methods. Gas chromatography-mass spectrometry and gas chromatography with nitrogen-phosphorous or electron-capture detectors have been also reported. Suitable methods for the separation of enantiomers of 2,3-BZs have been described. Thin-layer chromatography has been shown to be capable to isolate analytes from biological samples as urine or faeces. The reported chromatographic techniques are currently applied to define the metabolic pathways of 2,3-BZs in experimental and clinical studies.

Keywords: Reviews; 2,3-Benzodiazepines

Contents

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1. Introduction

The classical 1,4-benzodiazepines (BZs) and all other structure-related compounds have sedative and anxiolitic properties as well as muscle relaxant and anticonvulsant actions. Unfortunately, they have dependance capacity and the hazard of drug abuse. For this reason there is a growing need to find compounds with specific activity.

The 2,3-benzodiazepines (2,3-BZs) are structurally related to the classical BZs, but, in spite of their structural similarity, they differ significantly from the classical BZs in their pharmacological profiles.

The first described 2,3-BZ, i.e., Tofisopam (GYKI 52322), has been synthesized in 1974 by Kórösi and Láng [1]. Tofisopam showed a selective anxiolytic action without relaxant and anticonvulsant activities [2–5]. Other analogues of Tofisopam were further synthesized, i.e., Girisopam (GYKI 51189), Nerisopam (GYKI 52322) and GYKI 52466 [6,7]. Tofisopam (also known as Grandaxin®) and its derivatives are marketed by EGIS Pharmaceuticals (Budapest, Hungary), and Grandaxin® is currently used for therapeutic purposes as minor tranquillizers. These 2,3-BZs do not show affinity for the 1,4-BZ receptors [8–10]. Moreover, GYKI 52466 shows a different pharmacological profile with respect to Tofisopam, since it has muscle relaxant and anticonvulsant properties, acting as highly selective non-competitive antagonist at excitatory amino acid receptor, i.e., α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor [11–15]. The selective anticonvulsant activity showed by GYKI 52466 was proved against seizures induced by intracerebroventricular administration of AMPA in mice [16–18] and in two genetically epilepsy-prone animal models [19].

In the attempt to obtain a compound with peculiar anticonvulsant activity, a new series of 2,3-BZ-4-ones has been synthesized by Chimirri and co-workers [20]. The more active compound, i.e., the 1-(4′-aminophenyl)-derivative named as 2,3-BZ6 has been recently marketed as CFM-2 by Tocris Cookson (Ballwin, MO, USA). These 2,3-BZ-4-ones and the correspondent 2,3-BZ-4-thiones derivatives [21], have shown a more selective AMPA receptor antagonist action than GYKI 52466 [22–25].

LY300164 is another new 2,3-BZ proposed as anticonvulsant agent in animal models; it has been synthesized at Eli Lilly Laboratories (Earl Wood, UK) [26].

Chromatography plays a main role to investigate the pharmacokinetic pattern of these compounds in animals and humans as well as to determine their metabolic pathways. Moreover, chromatography has been applied for detection and quantification of impurities of drug substances, and to determine the enantiomer ratio of optically active 2,3-BZs.

This paper reviews the methods published in the scientific literature over the last 25 years for the determination of the 2,3-BZs.

2. Methods

2.1. Choice of references

Articles were searched by using of Chemical Abstract and the Medline database from 1974 through September 1999. Methods cited from these references or other methods available to the author were also included, where relevant, and covering the time period above.

2.2. Definitions and used terms

Standard abbreviations according to the style of this Journal have been used in this review. Abbreviations used are included in the list of non-standard abbreviations (Table 1). The 2,3-BZs are named according to their internationally-accepted non-proprietary names (INN), or their common names.

Table 1
List of non-standard abbreviations

ACN	Acetonitrile	HPLC	High-Performance Liquid Chromatography
BSTFA	*N,O*-bis-(trimethylsilyl)-trifluoroacetamide	IPA	Isopropanol
DAD	Diode-array detector	ISP	Ion spray
DCM	Dichloromethane	MeOH	Methanol
ECD	Electron capture detector	NPD	Nitrogen phosphorous detector
EI	Electron impact (MS)	NP-HPLC	Normal phase HPLC
EtOH	Ethanol	P	Plasma
FAB	Fast atom bombardment	PM	Post mortem
FID	Flame ionisation detector	RP-HPLC	Reversed phase HPLC
FSC	Fused silica column	RP-TLC	Reversed phase Thin-Layer Chromatography
GC	Gas Chromatography	SPE	Solid-phase extraction
GLC	Gas Liquid Chromatography	TMCS	Trimethylchlorosilane
GC–MS	Gas Chromatography–Mass Spectrometry	TMS	Trimethylsilyl

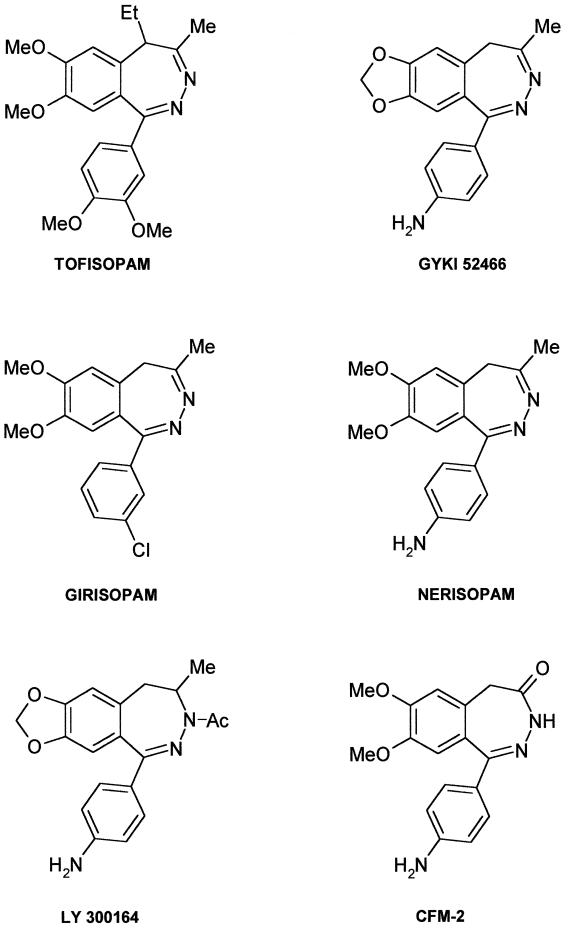

Fig. 1. Structures of Tofisopam, GYKI 52466, Girisopam, Nerisopam, LY300164, CFM-2.

3. Chemical structures

The 2,3-BZs are based on the 1-aryl-2,3-benzodiazepine structure characterized by a benzene ring fused on 10- and 11-positions of the 2,3-diazepine ring, with substituents in 7- and 8-positions. The aryl function at the 1-position is a phenyl with or without substituents. The structures are reported in Fig. 1.

Tofisopam is a 1-(3′,4′-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine; the analogue Girisopam is a 1-(3′-chlorophenyl)-4-methyl-7,8-dimethoxy-5H-2,3-benzodiazepine, and Nerisopam is a 1-(4′-aminophenyl)-4-methyl-7,8-dimethoxy-5H-2,3-benzodiazepine; GYKI 52466 is a 1-(4′-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine.

LY300164 is a 1-(4′-aminophenyl)-3-acetyl-3,5-dihydro-4-methyl-7,8-methylenedioxy-4H-2,3-benzodiazepine.

CFM-2 is a 1-(4′-aminophenyl)-3,5-dihydro-7,8-dimethoxy-4H-2,3-benzodiazepin-4-one. CFM-2, also known as 2,3-BZ6, and the 2,3-BZ-4-one analogues, are reported in Fig. 2.

4. Physico-chemical properties

4.1. Solubility and stability

2,3-BZs are basic in character and as neutral molecules are soluble in organic solvents such as methanol, ethanol, acetonitrile and diethyl-ether, slightly soluble in *n*-hexane, and almost insoluble in

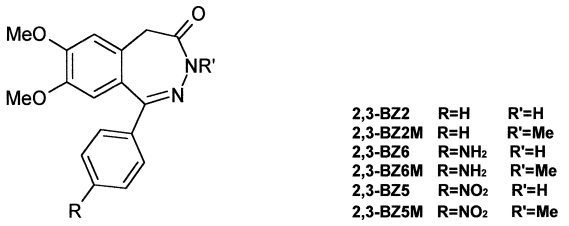

Fig. 2. Structures of 2,3-BZ-4-one series.

water. In spite of their low solubility in water, the 2,3-BZs are found to be totally soluble in the concentration range at which they appear in bioanalytical samples.

Usually, stock solutions of 2,3-BZs are stable in methanol and, more specifically, 10 μg/ml of working solutions are stable for 3 months under refrigeration [27]. Most of the 2,3-BZs undergo hydrolysis, particularly under acid or alkaline conditions, as it is well known for 1,4-BZs [28].

The physico-chemical properties of Tofisopam showed that its degradation is related to modification of pH value [29]. Valentik et al. [30] reported the behaviour of Tofisopam in different medium, such as 1 *M* NaOH, 1 *M* HCl and 1% H_2O_2 (10 volumes). Particularly, in H_2O_2, Tofisopam undergoes oxidative degradation producing two derivatives, i.e., monohydrazone and diketone, as recognized in the chromatogram by their retention time. In acid medium, Tofisopam undergoes hydrolysis and may produce the corresponding benzophenone that, in presence of NaOH, determines the formation of the fluorescent product, i.e., the naphthol derivative, used for spectrofluorimetric determination. Moreover, Tofisopam in dimethyl sulphoxide and in presence of NaOH may directly produce the naphthol derivative by heating [31].

Concerning Girisopam, as already mentioned for Tofisopam, its property to undergo an acid hydrolysis and aldol condensation in alkaline solution, producing a naphthol fluorescent derivative, has been exploited for spectrofluorimetric determinations [32].

4.2. Lipophilicity measurements

The biological activity of molecules is controlled by many factors, one of the most important being their lipophilicity. This parameter can de determined by the traditional method of partition between water and *n*-octanol, by using HPLC or reversed-phase thin-layer chromatography (RP-TLC). RP-TLC, a faster and low-cost technique, has been used to determine the lipophilicity of Tofisopam [33]; it has been shown that RP-TLC has a discrete predictive power in describing the HPLC retention behaviour concerning the lipophilicity of this substance.

The relative lipophilicity (R_m) of the 2,3-BZ-4-ones was measured by RP-TLC [34,35], and the 2,3-BZ-4-thiones showed a higher lipophilicity when compared with the corresponding isosters [20,21].

5. Specimen preparations

Because the 2,3-BZs are extensively and selectively bound to serum albumin [36], a deproteination process, using acid or organic solvent, is required to liberate the bound fraction of the drug from proteins. These drugs are usually present at trace levels (ng/ml) in a complex biological matrix, so the pretreatment of samples should be capable of concentrating the sample and reducing the amount of interfering substances. Despite of several attempts to avoid any sample preparation, the great majority of authors apply clean up and concentration procedures either on HPLC or GC. Moreover, when the biological samples are used for toxico-analytical studies, the samples should be pre-treated, since they are often limited in quantity and heavily contaminated [37].

The most common specimens used for the analysis of the reported compounds are serum/plasma, blood, urine and faeces. Blood, plasma and serum may be interchangeable in most methods, while urine requires hydrolysis prior to the isolation procedures. The biological samples can be kept for a few hours at room temperature, but for longer storage periods they should be frozen at −20°C. These conclusions have been drawn from studies in which the drug stability was assessed in replicate determinations of spiked plasma samples after storage [27,38–40]. Nevertheless, Eckstein [26] suggested to store the biological samples at −70°C, when the storage of samples should be done over 1 month.

Besides the biological samples, other specimens used to test the presence of drug impurities were the

tablets of Grandaxin® [30,41] and Tofisopam in methanol [33].

6. Extraction techniques

Chromatographic techniques, with few exceptions, require different procedures to separate and isolate the 2,3-BZs from biological matrices.

Three distinct approaches have been applied: liquid–liquid extraction; solid-phase extraction; direct injection into a chromatographic system.

6.1. Liquid–liquid extraction

Solvent used to extract the 2,3-BZs include methanol [27], chloroform [37,42], dioxane [41], dichloromethane [43], ethyl acetate [38,44]. These solvents have similar properties in extracting the substances, since they obtain recoveries well over 60% for all considered compounds.

Extractions are most commonly performed under slightly alkaline conditions by the use of sodium hydroxide [42–44]; near neutral conditions [27] or unbuffered condition [38–41] were also described. After extraction, the organic phase needs to be filtered in some circumstances [27,42]. During the analysis of lipophilic substances in a complex matrix, as body fluids, the conventional sample preparations are often associated with some disadvantages: the formation of emulsion, poor phase separation, high solvent consumption, low degree of automation. Liquid–liquid extraction, however, could become more efficient by using an hydrophilic stationary phase; such as a chemically inert matrix, able to retain aqueous samples, as the body fluids, but not lipophilic substances, as the 2,3-BZs. Consequently, the 2,3-BZs are eluted with an organic phase applied to the inert matrix. This approach allowed Rizzo et al. [44] to perform a simultaneous extraction of four 2,3-BZ6 derivatives from rat plasma, with a high and reproducible recovery. The inert matrix used was a wide-pore diatomaceous earth, marketed as Extrelut® (E. Merck, Darmstadt, Germany). The diatomaceous earth could be packed into glass columns of variable volume, in order to extract different kinds and volumes of biological samples. A stringent quality control of the final product guarantees uniform batch-to-bach quality, although the diatomaceous earth, as naturally occurring product, may be subject to certain fluctuations. Operating conditions are chosen so that the proteins and other interfering substances remain adsorbed on the inert matrix and the analyte migrates out of the column, by applying an organic eluent. This procedure should be considered a liquid–liquid extraction, since the analyte is collected in an organic phase, pure of interfering substances, and not retained by the inert matrix. Nevertheless, the diatomaceous earth is able to retain all the hydrophilic sample components. This method allows to isolate the 2,3-BZs in only one step and it should be considered to be the first choice method for extracting 2,3-BZs from biological samples. The Extrelut® 20 columns have been also used for isolation of metabolites from faeces (for up to 20 ml of sample) [38]; the procedures to obtain the free-metabolites include homogenization, purification on TLC and hydrolysis.

6.2. Solid-phase extraction

The solid-phase extraction (SPE) provides a more efficient and simple way of sample preparation than the traditional liquid–liquid procedure. Nevertheless, compared to liquid–liquid extraction on inert matrix, SPE is more expensive and time consuming. Solid-phase approach of extraction includes the use of sorbents as the reversed-phase high hydrophobic octadecylsilane-bonded cartridges (C_{18}), that well retain 2,3-BZs [26,38–40].

Rona [39] performed the SPE without internal standard, that was added only to the eluate after extraction; the calculated extraction efficiency ranged from 88% for Nerisopam to 90% for its *N*-acetyl metabolite.

Eckstein and Swanson [26] reported SPE of LY300164 from plasma of different species on mixed phase Bond-Elut Certify®; the accuracy of the extraction procedure was over 90%. Moreover, the extracts of the compound and its *N*-acetyl metabolite were stable for 24 h at room temperature.

C_{18} cartridges activated with methanol have been also used to extract 2,3-BZs from urine [38].

Octyl (C_8) bonded columns have been also successfully used with a good recovery for Tofisopam from human plasma [45].

6.3. Direct injection

In this method of analysis, the samples are directly injected into the HPLC column. Direct injection of complex samples, however, may lead to the contamination of columns impairing their performance. Contamination often persists even when a pre-column is used to protect the analytical column. Tomori et al. [38] described the on-line urine and serum analysis using a pre-column with a back-elution to the analytical column. Moreover, for further examination, appropriate HPLC fractions were treated properly, digested with glusulase (β-glucuronidase+ sulphatase), and rechromatographed by TLC and HPLC.

While this technique avoids an extraction step and thus, compared with other techniques, has the advantage to be less time-consuming, its use is limited by the fact that it could be only applied to HPLC.

7. Chromatographic techniques

There are three main chromatographic procedures used to separate and detect the 2,3-BZs and their related metabolites: HPLC, GC and TLC.

Chromatographic methods are currently used to determine 2,3-BZs in biological samples as well as to analyze the drugs in pharmaceutical preparations.

7.1. High-performance liquid chromatography

A summary of the chromatographic methods reviewed is reported in Table 2. Among the 14 studies reviewed, six dealt with plasma or serum [26,27,40,42–44]; one study was performed on plasma and urine [39], and another study dealt on plasma, urine and faeces [38].

The studies not dealing with biological samples were applied to the tablets of Grandaxin® [30,41], and to a methanolic solution of Tofisopam [33,46].

With the exception of two papers [38,41], in which serum extracts were chromatographed on normal-phase column, all other reviewed methods used reversed-phase separation. The more frequently chosen columns were the C_{18} cartridges, whereas the C_8 cartridges have been used only once [41].

Ultraviolet absorbance detection (UV) has been commonly used, but in some cases diode-array detection (DAD) has been preferred to supplement UV absorbance detection [30,38–40], since this detector offered real advantages in identifying peaks and establishing peak purity.

The limits of detection (LOD) ranged around 10 ng/ml. The LOD was dependent on the measured compounds (extinction coefficient), the volume of extracted specimens, the detection wavelengths and the used methods. A LOD lower than 3 ng/ml using 1 ml of serum was reported for Girisopam with UV detection at 235 nm [38]. This method, however, requires that almost all the extract to be injected into the column. Whenever possible, a larger volume of specimen (more than 1 ml) should be used. This is not the case of plasma obtained from rats: the volume of specimen is often less than 1 ml and thus, an extraction with a high recovery and a chromatographic method with adequate sensitivity is necessary. Ürmös et al. [40] elaborated a gradient RP-HPLC method to determine Girisopam and its four metabolites in humans and rat plasma. In humans, the LOD was 1 ng/ml for Girisopam and 2 ng/ml for its main metabolite. In rat plasma, the LOD was 10 ng/ml for Girisopam and 50 ng/ml for the metabolites. Recently, Rizzo et al. [27] reported a RP-HPLC method, validated on reproducibility, accuracy and specificity, using only 500 μl of rat plasma to determine two different 2,3-BZ-4-ones, i.e., 2,3-BZ2 and 2,3-BZ2M, with a LOD of 6.5 and 8 ng/ml, respectively. Moreover, by using a UV detector set at 240 nm, a RP-HPLC allowed the simultaneous determination of four different 2,3-BZ6 derivatives with a LOD ranging from 5.5 to 8.5 ng/ml [44].

Most of the authors used, as mobile phase, solvent/buffered solutions in isocratic way, or with ion-pairing reagents [30,39]. Methanol/unbuffered water may also be used [27]. Three methods used gradient programming [38,40,46].

Concerning the examined 2,3-BZs, Tofisopam has been the compound more extensively investigated. Saigo et al. [42] reported a liquid–liquid extraction of Tofisopam from human serum with an isocratic separation and UV detection: in this earlier study, the metabolites of Tofisopam did not appear in the chromatogram and, therefore, this method could apply only for determination of Tofisopam. The

Table 2
Summary of published HPLC methods

Ref.[a]	Matrix	Drugs	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	Detection limit[d]	Recovery[e]
[42]	Serum	Tofisopam	Chloroform 1 *M* NaOH ph 10	LiChrosorb Si-60 250×2.1 mm I.D. 5 μm	*n*-heptane/IPA/ MeOH (75:10:1, v/v) isocratic	1.0	UV 311	n.a.	34.6
[41]	Tablets	Grandaxin®	Dioxane/ ACN/MeOH (25:5:3)	NP-HPLC: Spherisorb S10 W 150×3 mm I.D. 10 μm	*n*-hexane/dioxane/ ACN/MeOH/25% (w/v) Ammonia (67:25:5:3:0.02, v/v)	0.83	UV 238	n.a.	99
				RP-HPLC: LiChrosorb RP-8 200×4.6 mm I.D. 5 μm	ACN/1% (w/v) ammonium carbonate/water (40:10:50, v/v)	0.83	UV 238	2.5	99
[33]	MeOH	Tofisopam	none	LiCrosorb RP18 250×4.6 mm I.D. 5 μm	ACN/0.06 *M* potassium diphosphate pH 4.8, gradient	n.a.	UV 230	n.a.	n.a.
[38]	Serum	Girisopam	Ethyl acetate	Metabolic study		1	UV 235	n.a.	n.a.
				Pre-column: LiChroprep RP8 40×4 mm I.D., 15–25 μm Column: Polygosil-60 C_{18} 250×4 mm I.D. 10 μm	A: MeOH/0.1 *M* ammonium acetate buffer pH 5.8 (3:7, v/v) B: MeOH/0.1 *M* ammonium acetate buffer pH 5.8 (4:1, v/v) gradient		DAD		
				Pharmacokinetic study		1	UV 235	3	60
				Polygosil-60 Si 250×4 mm I.D. 10 mm	*n*-hexane/EtOH/ diethyl ether (66:9:25, v/v) isocratic				
[30]	MeOH	Tofisopam	none	LiCrosorb RP-18 250×4.6 mm I.D. 10 μm	0.001 heptanesulphonic acid, sodium salt/ ACN/MeOH (46:31:23, v/v) isocratic	0.8	UV 230 DAD	n.a.	n.a.
[26]	Plasma	LY300164	Bond Elut SPE C_{18}	YMC basic 150×4.6 mm I.D.	MeOH/0.067 M citrate buffer/ACN pH 5.0 (40:50:10, v/v) isocratic	1	UV 310	50	93.3
[52]	MeOH	Tofisopam	none	Chiralcel-AGP 100×4 mm I.D. *T*=22–25°C	0,01 *M* phosphate buffer (pH 5.0, 6.0, 7.0) with 5% or 10% IPA or ACN	0,9	220	n.a.	n.a.
[43]	Blood	Tofisopam as I.S.	DCM/Buffer pH 8.0	C_{18} Microbore 250×1 mm I.D. 5 μm	ACN/2 m*M* NH_4COOH pH 3 (75:25, v/v) isocratic	0.5	ISP-MS	n.a.	n.a.
[39]	Plasma Urine	Nerisopam	Bakerbond SPE C_{18}	Precolumn: Nucleosil 7 C_{18} 30×4 mm I.D., 5 μm; Column: Nucleosil 7 C_{18} 250×4 mm I.D. 5 μm	2 m*M* heptanesulphonic acid with 0.04 *M* phosphoric acid/ACN/MeOH (70:25:5, v/v) isocratic pH 2.7 with ethylamine	1	UV 385 DAD	5	90
[46]	MeOH	Tofisopam	none	LiChrospher 100 RP18 200×4.6 mm I.D. 5 μm	MeOH/water gradient	Various	UV	n.a.	n.a.

Table 2. Continued

Ref.[a]	Matrix	Drugs	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	Detection limit[d]	Recovery[e]
[51]	MeOH	Tofisopam	none	Chiralcel OF 460×25 mm T=22–40°C	A: hexane/2-propanol (50:50, v/v) B: hexane/2-propanol/diethylamine (50:50:0.1, v/v) C: hexane/absolute EtOH (50:50, v/v)	0.7	240	n.a.	n.a.
				Chiralcel OJ 460×25 mm T=22–40°C	A: hexane/2-propanol (50:50, v/v) B: hexane/absolute EtOH (50:50, v/v)	0.7	240	n.a.	n.a.
[40]	Plasma	Girisopam	SPE	Hypersil BDS C_{18} 250×2 mm I.D. T=40°C	A: ACN/MeOH/water/ammonium carbonate/ammonium chloride (20:12:80:0.1:0.08, v/v) gradient B: ACN/MeOH/water/ammonium carbonate/ammonium chloride (18.5:11:20:0.03:0.04, v/v) gradient	0.55	238 DAD	1 Human P 10 Rat P	90
[27]	Plasma	2,3-BZ2 2,3-BZ2M	MeOH	Ultrasphere ODS 250×4 mm I.D. 5 μm	MeOH/water (35:65)	1.5	UV 254	6.5 8	98
[44]	Plasma	2,3-BZ6 2,3BZ6M 2,3BZ5 2,3BZ5M	Extrelut®1 Ethyl acetate	Partisil 10 ODS 250×4 mm I.D. 10 μm	ACN/acetate buffer pH 5.25	2	UV 240	5.5–8.5	93.75

[a] References are chronologically listed.
[b] Flow rate in ml/min.
[c] Wavelengths in nm.
[d] Detection limits in ng/ml.
[e] Recoveries in per cent.
n.a.=Not available or not described.

simultaneous determinations of Tofisopam and its impurities in Grandaxin® tablets, performed by both normal and reversed-phase HPLC, were superior to TLC and GC in terms of efficiency and loadability [41]. Nevertheless, in another study [33] the determination of Tofisopam by TLC showed a good correlation with HPLC assay. Tofisopam determination by HPLC may be implemented by a statistical technique of optimizing mobile phases for HPLC, defined as overlapping resolution map (ORM), in order to obtain the fastest analysis compatible with the desired separation [46]. The use of Tofisopam as internal standard (I.S.) has been suggested for HPLC–MS determination of colchicine in plasma [43]. The method used a C_{18} Microbore column and a ion-spray mass spectrometry (ISP-MS). The mass spectrometer instrument was used in the positive ionization mode. The fragments were collected using either total ion current (TIC) or in the selected ion monitoring (SIM) mode. Tofisopam has been chosen as I.S. because it presented a molecular weight similar to colchicine, allowing the use of a narrow TIC and resulting in an improved sensitivity. Moreover, Tofisopam as I.S. could be co-extracted with colchicine from the biological fluid by a single-step liquid–liquid extraction. The combination of HPLC with MS is a useful tool for the analytical study of 2,3-BZs, since this combination provides the separation power of HPLC and the sensitivity and specificity of MS.

Concerning other 2,3-BZ compounds different from Tofisopam, other HPLC methods have been

reported. Girisopam has been investigated by using normal-phase chromatography on a silica column [38] as well as by using reversed-phase chromatography with gradient programme [40].

Nerisopam and its *N*-acetyl metabolite were simultaneously detected in human plasma with a RP-HPLC method suitable for pharmacokinetic studies and human drug monitoring [39].

LY300164 and its metabolites were studied by RP-HPLC in isocratic way using UV detection. The sensitivity of the method allowed the investigation of the pharmacokinetics of this compound in preclinical studies. The lower limit of quantitation (LOQ) was 0.05 μg/ml in plasma of all tested species; the LOQ for its metabolites was 0.05 μg/ml in dog and monkey plasma, and 0.1 μg/ml in mouse and rat plasma [26].

CFM, formerly known as 2,3-BZ6, has been analyzed by a fully validated RP-HPLC assay, showed a large linear range, with an accuracy for all compounds ranging from 92 to 105.5%. The chromatography has shown to be selective and allowed the simultaneous determination of the drug and its derivatives, i.e., 2,3-BZ6M, 2,3-BZ5 and 2,3-BZ5M [44].

7.2. Gas chromatography and gas chromatography–mass spectrometry

A summary of the reviewed chromatographic methods is reported in Table 3. GC studies usually were performed on fused-silica columns (FSC) [38,45,47,48]. The type of column ranged from non polar vinyl [38], or slightly polar 5% phenyl methyl silicone [45] and 100% methyl–silicone phase [47], to the polar cyanoethyl [48] and cyanopropylmethyl–phenylmethyl phase [38]. The use of dimethyl silicone stationary phase gave better separation of Tofisopam metabolites than the cyanoethyl phase [48].

All the methods reviewed did not use the temperature programming, unimportant to detect compounds with small polarity differences.

The most common used detectors have been nitrogen-phosphorous detector (NPD) and electron-capture detector (ECD); ECD showed the best detection limits compared to NPD. Gaillard et al. [45] studied a rapid twin-column GC method for simultaneous determination of Tofisopam and other BZs commonly used in therapy. Two identical fused-silica columns (5% phenyl methyl silicone) were connected to NPD and ECD to compare the performance of these detectors. ECD afforded a higher degree of selectivity and sensitivity. The method is appropriate for the quantification of Tofisopam in a single/run within 30′, and a LOQ of 2 ng/ml is valuable for toxokinetic and pharmacokinetic studies, and moreover for monitoring drugs. The flame ionization detector is not enough sensitive; Benko et al. [47] reported a detection threshold of only 1 μg.

Mass spectrometry applied to GC has been used to identify the unknown structures, i.e., 2,3-BZs metabolites. Derivatisation of Tofisopam, using BSTFA/TMCS as silylating reagents to produce trimethylsilyl (TMS) derivatives, was preferred by Tomori et al. [48], which afforded better thermal stability and produced derivatives with well defined mass spectra. Nevertheless, in the presence of a bulky TMS group, the separation of the compounds was insufficient when they were analysed without derivatisation on dimethyl silicone phase.

GC and HPLC have been used as complementary techniques in a metabolic study of Girisopam. Indeed, the metabolites were separated by HPLC and GC; particularly, GC was used to find minor metabolites in urine. The structures of metabolites were determined by GC–MS; the fast atom bombardment method was used to provide mass information and to confirm the structures of metabolites [38].

7.3. Thin layer chromatography

A summary of the reviewed chromatographic methods is reported in Table 4. TLC was the first developed chromatographic technique. Traditionally, BZs have been measured as benzophenones, formed after acid treatment of extracts. The TLC has been successfully applied to separate 2,3-BZs and related metabolites from biological samples, such as post mortem stomach and intestine contents [37], urine and faeces [38,49], or urine alone [48].

Nonetheless, since most of 2,3-BZs urinary metabolites are conjugated with glucuronic acid, the urinary samples should be usually analyzed by TLC before and after hydrolysis.

Tomori et al. [48] studied urinary Tofisopam

Table 3
Summary of published GC and GC–MS methods

Ref.[a]	Matrix	Drugs	Extraction method	Column conditions	Derivitisation method	Detection method	Detection limit[b]	Recovery[c]
[47]	PM	Tofisopam	TLC	OV-101 on Gas Chrom Q N_2 GC, $T=310$°C isothermal	None	FID	1000	n.a.
[48]	Urine	^{14}C-labelled Tofisopam	Chloroform TLC Enzymatic hydrolysis	A: 2% XE-60 on Gas-Chrom Q N_2 GLC, 45 ml/min, $T=240$°C isothermal	None	FID	n.a.	n.a.
				B: 2% OV-101 on Gas Chrom Q N_2 GLC, 25 ml/min, $T=200$°C isothermal	None	FID	n.a.	n.a.
				B: 2% OV-101 on Gas Chrom Q N_2 GLC, 45 ml/min, $T=200$°C isothermal	BSTFA/ TMCS	EI–MS	n.a.	n.a.
[38]	Serum	Girisopam	Ethyl acetate	2% OV-225 on Gas Chrom Q GC argon/methane (95:5), 50 ml/min $T=225$°C isothermal	None	Ni-ECD	3	60
				3% OV-1 on Gas Chrom Q GC argon/methane (95:5), 50 ml/min $T=240$°C isothermal	None	FAB-MS	n.a.	n.a.
[45]	Plasma	Tofisopam	SPE C_8 0.5% Acetic acid in MeOH	Ultra 2 GC Helium 2.1 ml/min $T=210$°C isothermal	None	NPD/ECD Dual Channel	2	86

[a] References are chronologically listed.
[b] Detection limits in ng/ml.
[c] Recoveries in per cent.
n.a. = Not available or not described.

metabolites extracted with an organic solvent and separated using preparative TLC. Hydrolysis has been realized with glusulase. Moreover, analysis of sample aliquots, scraped off the plates, were done using the GLC–MS.

As already mentioned, TLC may be used to predict the HPLC retention behavior of 2,3-BZs and related metabolites. Indeed, Valko et al. [33] reported the reversed-phase retention behaviour of Tofisopam using TLC and HPLC and found that in RP-TLC the R_m value depended linearly on the organic phase concentration, and logarithmically on the buffer concentration of the eluent. Moreover, they demonstrated a highly significant correlation between the HPLC log k_0 value and RP-TLC parameters, even if the TLC parameters explained only 75% of the total variance, thus limiting the predictive value of TLC with respect to HPLC retention behaviour.

TLC may be used to control the chemical purity of labelled compounds used for metabolic studies. Urine and faeces were collected from animals treated with 5-ethyl-labelled form of Tofisopam. Metabolites

Table 4
Summary of published TLC methods

Ref.[a]	Matrix	Drugs	Extraction	Support	Mobile phase	Detection method[b]
[37]	Stomach/ Intestine contents (PM)	Tofisopam	Chloroform in acid medium	Silica Gel GF_{254}	Cyclohexane/benzene/diethylamine (75:15:10, v/v); visualization with Marquis reagent	UV 311
[49]	Urine	^{14}C-labelled Tofisopam	Chloroform	Kieselgel 60 F_{254}	Benzene/EtOH/cyclohexane (1:1:1, v/v)	n.a.
[48]	Urine	Tofisopam	Chloroform	Silica gel-PF-60_{254}	Chloroform/*n*-hexane/EtOH/ conc. ammonia (40:40:20:3, v/v)	UV 254
[33]	MeOH	Tofisopam	none	Silicoplat F_{254}	ACN in gradient with potassium diphosphate at different molarity	UV 230
[38]	Urine	^{14}C-labelled Girisopam	Samplex C_{18}	Kieselgel 60 F_{254}	MeOH/water (3:2, v/v)	Autoradiography
	Faeces		Extrelut® 20	Kieselgel 60 F_{254}	MeOH/water (3:2, v/v)	Autoradiography

[a] References are chronologically listed.
[b] Detection limits in ng/ml.
n.a. = Not available or not described.

were isolated from the samples by chloroform and separated by preparative-TLC. The isolated metabolites were purified by re-chromatography and the structures were determined by mass spectrometer [49].

Besides the traditional TLC, radio-TLC has been performed on digested faeces samples for the investigation of metabolites of Girisopam in humans [38].

7.4. Chiral separations

Tofisopam is a 2,3-BZ having a chiral centre at the C(5) position and existing in solution in two conformations; the conformers of the same enantiomer show opposite optical rotation, as it has been proved by NMR [50]. The majority of the molecules have a conformation in which the C-5 ethyl group is pseudo-equatorial, while in the remaining molecules this group is pseudo-axial with respect to the diazepine ring. In crystalline form, the molecules are predominantly in the thermodynamically more stable conformation, i.e., the C-5 ethyl group in pseudo-equatorial position. In solution, molecules exist in two conformations which slowly interconvert into each others and equilibrium is achieved within a few hours.

The binding of Tofisopam enantiomers to human serum albumin (HSA) has been investigated by ultrafiltration and affinity chromatography. On an HSA-Sepharose column it is possible to separate the major conformers of the two enantiomers as well as the conformers of the (*S*)-enantiomer [36].

Bidló-Iglóy [51] described a chiral separation by HPLC of optically active 4,5-dihydro-2,3-BZ derivatives. The chiral separation was performed by using a cellulose tribenzoate derivative (Chiralcel OJ), or a cellulose triphenylcarbamate derivative (Chiralcel OF), as chiral stationary phases. On Chiralcel OF a higher degree of resolution was obtained in the analysis of molecules containing an aromatic NO_2 or NH_2 group.

Moreover, chiral separation may be improved by the addition of an organic modifier to the mobile phase. Indeed, Fitos et al. [52] reported the separation of *rac*-tofisopam and its enantiomers obtained on an α_1-acid glycoprotein chiral column, at pH 7.0, with 5% isopropanol (IPA), or 5% acetonitrile

(ACN) modifiers; when the ACN modifier was used, the conformers of both enantiomers could be better separated than by using IPA.

8. Metabolism and pharmacokinetic data

In most cases, the administered drug is the compound responsible for the pharmacological action; however, there are cases in which the activity is due to some of the metabolites produced in the organism [24,25]. In these cases, the administered drug is a precursor of the pharmacologically active compound.

The metabolic pathway of 2,3-BZs has been extensively studied in different species.

Tofisopam metabolites have been detected in animals [5,48,49] as well as in humans [5,48,53,54]. Elekes et al. [49] reported the pharmacokinetic properties and the metabolism of Tofisopam using the ^{14}C-labelled form of the molecule, in rats. The orally administered drug was rapidly absorbed in the gastrointestinal tract. The maximum blood concentration (C_{max}) was detected 60 min (T_{max}) after treatment. Radiochromatographic studies revealed the presence of the compound in the brain in its original form. Tomori et al. [48] reported that the major pathway of the metabolic transformation of Tofisopam is demethylation; four different monodemethylated metabolites were identified in urine of humans and different species of animals.

The pharmacokinetic profile of Tofisopam can be described by a two compartment open-model, with a rapid absorption and distribution phase [5]. The Tofisopam unchanged aliquot is eliminated from human plasma slower than the metabolites. The main route of elimination is the excretion of the mainly conjugated metabolites after the intensive first-pass metabolism in urine and/or faeces. The major route of biotransformation is mono-, di-, tri- and tetra-*o*-demethylation in various degrees and positions of aromatic rings.

Concerning Girisopam, Tomori et al. [38] reported that, after a 100 mg oral dose of ^{14}C-labelled Girisopam, the mean recovery of ^{14}C radioactivity was 51% in urine and 33% in faeces. Girisopam was rapidly absorbed, with a T_{max} of 2 h and a mean elimination half-time of 22 h. The metabolic profile in the serum consisted predominantly of the glucuronides of 7-demethylgirisopam, 4′-hydroxygirisopam and 4-hydroxymethyl-4-demethylgirisopam. The major urinary metabolites were the same as in serum, which were in conjugated form; and 4-carboxy-4-demethylgirisopam, a compound with an open-chain structure, which was in non-conjugated form.

Nerisopam and its *N*-acetyl metabolite have been detected in humans [39]. Nerisopam was *N*-acetylated through polymorphic metabolism. In particular, volunteers belonging into slow or fast acetylating phenotypes showed significant different plasma concentrations. Observed pharmacokinetic differences were primarily manifested in the absorption phase. Accordingly, slow acetylators had higher Nerisopam levels, while fast acetylators possessed higher metabolite levels.

Moreover, it has been shown that Nerisopam underwent significant ''first pass'' metabolism process, the extent of which was different between the two acetylator phenotypes [55].

In rats, the metabolism of Nerisopam could be described by a two-compartment open model. The *N*-acetyl metabolite of Nerisopam showed a higher C_{max} than Nerisopam, and the elimination of the metabolite was concentration-dependent; the metabolism of the *N*-acetyl metabolite could be described by a one-compartment open model [56].

The metabolic pathway of 2,3-BZ-4-ones has been described by Rizzo et al. [27]; the N3-methyl derivative, i.e., 2,3-BZ2M, underwent a N3-demethylation after administration in rats. The peculiar metabolism of CFM-2 derivatives has been reported, more recently, by the same authors [57]. The pharmacokinetic study demonstrated that 2,3-BZ6M, 2,3-BZ5 and 2,3-BZ5M derivatives, also named as CFM-3, CFM-4 and CFM-5 respectively, achieved a C_{max} between 45 and 75 min. Moreover, the time profiles of plasma concentration showed a biotransformation of these 2,3-BZ-4-ones into a CFM-2, through two different metabolic pathways. The N3-methyl derivatives CFM-3 and CFM-5 underwent a N3-demethylation; moreover, CFM-5 as the other 4′-nitrophenyl derivative CFM-4, underwent a nitroreduction of the 4′-substituent of the aryl ring in C-1. The CFM-2 analogues are prodrugs of CFM-2, in which they are

biotransformed via the above-mentioned metabolic pathways.

9. Conclusion

There are a number confirmation tests available for 2,3-BZs. Most favoured in term of number of publications in the time period under review were HPLC techniques, although both GC and TLC techniques were also strongly represented.

HPLC techniques were capable of detecting most of the reported compounds using UV absorbance detection, although DAD detection provided more informations in detecting unknown peaks. There was no obvious preference for column choice, except reversed-phase C_{18} columns which were more prevalent than the alternative phases. Isolation steps were almost evenly distributed between conventional liquid–liquid extraction and solid-phase extraction procedures. A new prospective has been represented by the combination of HPLC with MS which offer an important alternative to conventional GC–MS.

GC methods using fused-silica capillary columns were most commonly used with nitrogen-phosphorous detector, although some papers found electron-capture detector as a more useful alternative. Slightly polar phases, as 5% diphenyldimethylsiloxane phases, were preferred for best separation capability.

GC–MS methods were also reviewed allowing, as expected, interesting results in metabolism study. Derivatisation improved spectral definition and reduced thermal degradation on-column. Fast atom bombardment measurement was used to confirm the structures of 2,3-BZ metabolites.

TLC has shown little interest in the more recent publications because of lack of sensitivity and specificity. This technique may be used to predict the HPLC retention behavior and, to isolate metabolites from the biological samples.

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