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Cent. Eur. J. Chem. • 12(10) • 2014 • 994-1007
DOI: 10.2478/s11532-014-0551-1

Central European Journal of **Chemistry**

Analytical methods for determination of benzodiazepines. A short review

Review Article

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Received 16 July 2013; Accepted 6 February 2014

Abstract: Benzodiazepines (BDZs) are generally commonly used as anxiolytic and/or hypnotic drugs as a ligand of the $GABA_A$-benzodiazepine receptor. Moreover, some of benzodiazepines are widely used as an anti-depressive and sedative drugs, and also as anti-epileptic drugs and in some cases can be useful as an adjunct treatment in refractory epilepsies or anti-alcoholic therapy. High-performance liquid chromatography (HPLC) methods, thin-layer chromatography (TLC) methods, gas chromatography (GC) methods, capillary electrophoresis (CE) methods and some of spectrophotometric and spectrofluorometric methods were developed and have been extensively applied to the analysis of number of benzodiazepine derivative drugs (BDZs) providing reliable and accurate results. The available chemical methods for the determination of BDZs in biological materials and pharmaceutical formulations are reviewed in this work.

Keywords: *Analytical methods • Benzodiazepines • Drugs analysis • Pharmaceutical formulations*

1. Introduction

Benzodiazepines have been first introduced into medical practice in the 60s of the last century. They represent one of the most widespread classes of drugs used primarily for the treatment of psychiatric disorders, anxiety and insomnia. The first synthesized benzodiazepine was chlordiazepoxide obtained in 1957 in a Hoffman La Roche laboratory, and after two months of laboratory testing the sedative effect of this compound was confirmed, which resulted in its introduction to the treatment in 1960 under the trade name Librium. In the following years, many structural derivatives of chlordiazepoxide were synthesized as for example oxazepam and diazepam. These compounds showed much stronger sedative effect in comparison to the original ones.

Furthermore, targeted synthesis allowed to the production of other benzodiazepines with characteristic hypnotic and/or anticonvulsant functions, and different biological half-life (half-life of drugs is described as short, medium and long). For this reason, an application of these drugs became broader allowing their utility to a larger extent, and at the same time, problems related to drug abuse were reported [1-5]. Therefore, it is necessary to apply fast and accurate detection method of these compounds.

2. Chemical structure, selected physicochemical properties, mechanism of action, metabolism and pharmacokinetics of benzodiazepines derivatives

In terms of the chemical structure benzodiazepines can be divided into five main groups [1,5-8]:

1. Derivatives of 1,4-benzodiazepine (the largest group of benzodiazepines as chlordiazepoxide, diazepam, clonazepam, lorazepam, oxazepam),
2. Derivatives of 1,5-benzodiazepine (clobazam, triflubazam),

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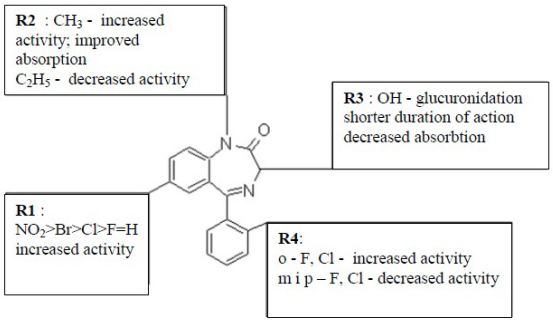

Figure 1. Relationship between chemical structure of 1,4-benzodiazepin-2-one derivatives and strength of their activity [9].

3. Tricyclic derivatives called triazolobenzodiazepines (alprazolam, adinazolam, estazolam, loprazolam, triazolam),

4. Derivatives of thienodiazepine (brotizolam, clotiazepam),

5. Imidazolobenzodiazepines (midazolam).

Generally, the chemical structure of benzodiazepine consists of seven-atom rings attached to various aromatic structures, and a certain moiety, which may be modified without loss of compound's biological activity (see Fig. 1) [9-10].

The largest group of benzodiazepines is the group of 1,4-benzodiazepine derivatives, especially 1,4-benzodiazepin-2-one derivatives, for which relationship between the structure and activity of these compounds can be characterized. Carbon substituted at position 7 in atom ring plays an important role in determination of activity for the wide spectrum of benzodiazepine derivatives. Substituents such as Br, Cl, F, enhance compound's activity. In this group, commonly used drugs, possess a halogen atom or a nitro group in this particular ring position. The chlorine-substituted atom at position 7 provides anxiolytic and sedative properties of the compound. The substitution of hydrogen atom by bromine atom does not significantly affect pharmacological activity of drugs. In turn, 7-nitrodemetylodiazepam derivatives, for example nitrazepam, flunitrazepam and clonazepam, are used as hypnotic and antiepileptic drugs. Relationship between chemical structure of benzodiazepine and its activity is presented in Fig. 1 [9-12].

Introduction of methyl groups instead of nitrogen at position 1, observed also in diazepam, increases their biological activity and facilitates absorption from the gastrointestinal tract, as compared to compounds without functional groups mentioned above. Drugs substituted at N1 position such as halazepam, pinazepam, prazepam, and flurazepam also show increased lipophilicity. An important characteristic of these derivatives is their metabolism and their ability to influence the long half-life of active metabolites formation. One of them, demetylodiazepam was introduced as a drug designated as nordazepam [1,9]. However, one should also mention benzodiazepine-similar structure compounds and their selective antagonists for example flumazenil-imidazobenzo-1,4-benzodiazepine derivative [1-2]. It is characterized by the presence of C-keto functional group, methyl substituent, and fluorine at position 5, 4, 7, respectively in the benzene ring. Phenyl ring associated with agonist activity was not reported [1,8].

Activity of benzodiazepine derivatives affects different parts of human brain, for example cord (muscular atony), cerebellum (ataxia), brain stem (hypnotic and antiepileptic activity), visceral brain and cortical cortex (emotional reaction). Initially, it has been thought that benzodiazepines act as non-specific depressants affecting central nervous system (CNS) [2]. However, further study showed, that all of these compounds used in clinical practice are capable of potentiate selective binding of γ-aminobutyric acid (GABA), (the primary neurotransmitter inhibitor), to $GABA_A$-subtype receptor and properly to the GABA-chloro-benzodiazepine receptor complex [5,7,8]. Complex formation strengthens pharmacological activity of benzodiazepine derivatives related not only to their anxiolytic activity, but also sedative and hypnotic effect, skeletal muscle relaxant and anticonvulsant activity [5]. $GABA_A$ receptors are located postsynaptically and they are involved in postsynaptic inhibition [2]. Molecular cloning methods and recombinant receptor-based research has demonstrated huge diversity, which is a consequence of different RNA splicing variants manifested by the presence of 16 types of subunits, classified as seven subfamilies: α (1-6), β (1-3), γ (1-3), δ (1), η (1), ε (1), θ (1) and Π (1) [5,7,8]. $GABA_A$ receptors are composed of five segments with three different subunits (α, β, γ), where α-forming several loops around central ion channel with larger fragment located extracellular and containing binding site of GABA [2]. The term α subunit is synonymous with benzodiazepine receptor (BZ receptor), because it can affect the ability to bind benzodiazepines [5,13,14]. Benzodiazepines act in binding target sites as agonists by allosteric interaction, increasing GABA binding level and thereby intensifying the strength of action. However, in the absence of γ-aminobutyric acid, they have no effect on $GABA_A$ receptor activity [8,14]. Indirectly, they decrease GABA affinity to $GABA_A$ receptor complex with chloride channel, increasing frequency, but not the chloride channel opening duration, so that an increased amount of chloride ions can penetrate under a concentration

gradient into the cell. Activation of postsynaptic $GABA_A$ receptors leads to hyperpolarization of cell membrane with subsequent decrease in the degree of cell excitability due to decreased sensitivity of this receptor to excitation pulse. It is one of the main inhibition pathways of neurons in the brain structures [8,9,14]. Benzodiazepines are used as muscle relaxants acting centrally, decreasing skeletal muscle tension by the inhibition of multisynaptic impulses without a crucial effect on neuromuscular signaling transduction pathway in motor endplate [14]. The use of sedative drugs stimulates GABA-ergic signaling pathways, which regulate the stimulation of neuronal firing response and releasing various monoamines [8]. In the cortical cortex, GABA-ergic interneurons exert an inhibitory effect on pyramidal neurons, leading to reduced excitation of neurons maintaining induced anticonvulsant effect [5,8].

Common characteristic of all benzodiazepines is associated with their slightly elimination by kidneys in unchanged form (1-2%); and a fact, that, C3 hydroxylation leads to an active metabolite formation [5,9]. These compounds are metabolized in liver and excreted in the urine as glucuronide conjunctions (approximately 60%). To some extent, they are eliminated through the digestive system [2,4-5]. Metabolism is closely associated with cytochrome P-450 subfamily enzymes and relates mainly to oxidative phase transition. For example, CYP3A4 was reported to be involved in the metabolism of clonazepam, temazepam and triazolam. Moreover, metabolism of diazepam and flumitrazepam were reported to be catalyzed by CYP 3A4 and CYP 2A19 [9,15]. However some of benzodiazepines such as lorazepam, lormetazepam, temazepam and oxazepam are not metabolized in this manner, but are directly conjugated with glucuronic acid [6-8]. Others, in turn, are metabolized in so-called 'first pass effect' way, which means that these drugs are extensively metabolized after absorption, and their metabolites such as midazolam and triazolam, have psychotropic activity [7].

Metabolism of benzodiazepines is characterized by three basic steps. In case of compounds with modified moiety at 1 or 2 position of diazepine ring, first phase has the highest efficiency and involves the modification of metabolism and/or removal of that group [8]. Many biologically active metabolites are formed during this phase. The vast majority of those metabolites have longer duration time in comparison to parent drug. A good example is represented by demetylodiazepam (nordiazepam), which is a common metabolite of diazepam, clorazepate, halazepam and prazepam [5,8]. Its half-life of approximately 60 hours determines the accumulation of this compound in the body and its prolonged activity [2]. Metabolite compounds may also have a biological half-life similar to parent compound, *e.g.* α-hydroksyalprazolam [9]. For this type of drugs, clinical effectiveness is the result of the drug and its metabolites activity, which may contribute to difference in the operation of single-and multiple-dose [5]. The second phase involves metabolic hydroxylation at position 3, resulting in the formation of an active derivative. This process occurs slower than first phase, without problems associated with the accumulation of hydroxylated products from unmodified groups at position 1 (with the exception of diazepam after chronic use). The last step involves in the conjugation of the 3-hydroxy compounds mainly with glucuronic acid, with pharmacologically inactive product as a result [8]. The metabolism of benzodiazepines decreases with age, thus the elderly that takes these drugs, are characterized by shifted metabolism through oxidation rather than conjugation. This process observed increases with age and may cause increase compounds' half-life and consequently- sleepiness [2]. Moreover, under reduced hepatic blood flow, for example in liver diseases, metabolism of benzodiazepines is slower. In this case it is recommended to use drugs (oxazepam, lorazepam, temazepam), which do not form active metabolites [4]. Metabolism of benzodiazepines may decrease when they are taken in parallel with drugs inhibiting the activity of cytochrome P-450, such as cimetidine, oral contraceptives, and macrolide antibiotics (erythromycin) [7-8]. From practical point of view, the difference in metabolism of these drugs occurs between races and may result from mutations in CYP 450 2C19 genes associated with the decrease of oxidation and N-demethylation of diazepam [7]. Transformation of benzodiazepines during enzymatic hydrolysis is presented in Fig. 2.

Benzodiazepines demonstrate different pharmacokinetics, *i.e.*, potency, receptor selectivity, lipophilicity, metabolism of active derivatives and blood protein binding [7]. Benzodiazepines are well absorbed after oral administration providing maximum concentration in plasma after 1-4 hours [8]. This process occurs immediately (1 h) in terms of highly lipophilic and short acting compounds such as triazolam, which is used as a hypnotic drug [7]. After following intravenous administration, benzodiazepines are redistributed as characteristic substances with high solubility in lipids, which result in immediate central effect. However the effect disappears quickly since drug is transported to other tissues [8]. Diazepam in the treatment of epilepsy or midazolam used in general anesthesia, are good examples of compounds with this type of activity. After intramuscularly application benzodiazepines

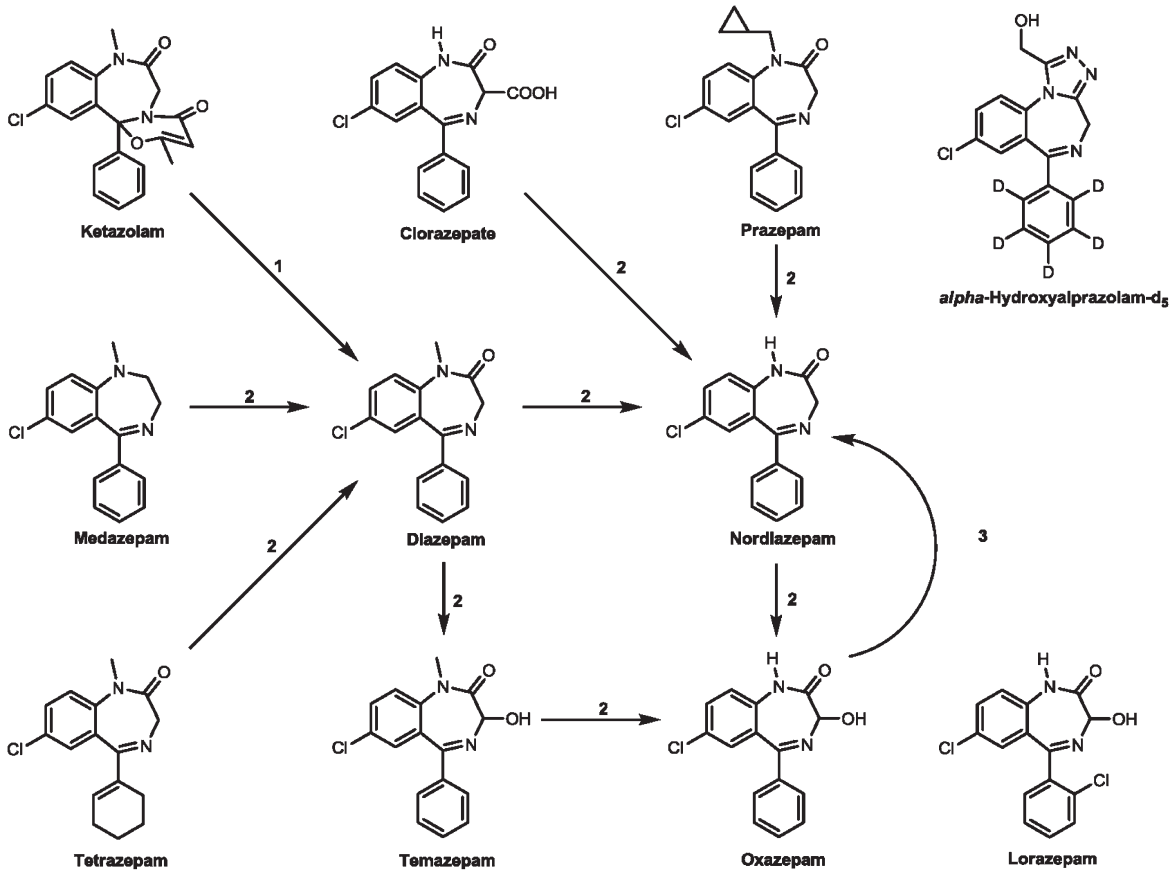

Figure 2. Transformation of benzodiazepines during enzymatic hydrolysis.

are absorbed very slowly [2]. Lipophilicity is important for their absorption, or their ability to cross biological barriers or penetrate to the central nervous system [5]. All benzodiazepines in the non-ionized form represent high lipid-water partition coefficient. This property depends on their polarity and electronegativity [8]. Benzodiazepines and their active metabolites are capable of binding plasma proteins. The bond strength correlates with their lipid solubility, ranging approximately from almost 99% for diazepam to about 70% for alprazolam [8]. It should also be underlined that only free form of compound, which is not bound with plasma proteins, demonstrates biological activity [5]. The pharmacokinetic properties, mentioned above, such as the ability to bind plasma proteins, and good solubility in lipids make these compounds capable of accumulation in adipose tissue. These two properties also affect distribution volume, which in general represents large values increasing with age [2,8]. Benzodiazepines also have the ability to penetrate placenta, cerebrospinal fluid, and maternal milk, with the concentration equal to that from blood serum [7,8].

3. Analytical methods used for the determination of benzodiazepines

3.1. Liquid chromatography

Liquid chromatography methods include those techniques in which liquid is used as a mobile phase. We distinguish liquid chromatography (LC) - the oldest among the chromatographic methods, and also modern techniques, such as high-pressure liquid chromatography known also as high-performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) [16]. HPLC methods are applicable to both qualitative and quantitative analysis of various compounds and mixtures. Qualitative analysis is based on separation and identification confirming the identity of analyzed compounds, complex mixtures or pure drug [17,18]. These techniques are widely used not only in the pharmaceutical industry, but also in toxicological analysis, control of doping in forensic medicine, agriculture and food industry [19]. High-

performance liquid chromatography (HPLC) has been widely applied to analysis of benzodiazepine derivatives mainly in biological fluids such as urine [19-40], hair [24-26,30,32,41-44], plasma [28,34,38,45-49,50-52,53-65,66-71], serum [35,36,40,52,70,72-76], whole blood [23,24,34,73,74,76-81], nails [43], gastric fluids [34], tissue fragments [34] and oral secretion fluids [25-27,32,82-85] as well as in pharmaceutical preparations [33,86-89], *i.e.*, tablets [90-98] capsules [97-100] and injection solutions [101].

After proper sample preparation, the HPLC method also allows for the identification and determination of benzodiazepines in pharmaceutical complexes as well as in other medical substances [79,92,94,95]. It is also used for the detection of contaminants in pharmaceuticals containing the benzodiazepine derivatives [97], studies about the stability of benzodiazepines in certain formulations [98] and separation of the enantiomers [102,103]. In the case of biological fluid, the HPLC method also enables identification of metabolites such as glucuronides [35,55]. Authors are used to utilize many different extraction methods for benzodiazepine analysis from biological samples, for instance: solid phase extraction (SPE) [19,20,31,37,44,47,48,50,52,55,60,64,72,82,104] utilizes Oasis HLB columns [21-22,29,71], BondElut Certify [42], Varian Bond Elut, [83] or columns modified with cyanopropyl groups [56]; liquid – liquid extraction (LLE) using different solvents [23,25,26,30,34,36,39,41,45,46,54,57,59,63,67,77,80,84,85,105]; solid phase microextraction (SPME) [40,72,106]; SPME utilizing alkyl-diol-silica coated wall (ADS) [75]; moleculary imprinted polymer solid phase extraction (MISPE) [44].

In a practical point of view, reverse phase HPLC (RP-HPLC) [33-35,51,52,54,56,58,59,62,63,71,74,80,81,90-93,95,96,98,100,107,108] is more frequently utilized than, normal phase HPLC (NP-HPLC) [67]. Typical RP column include C18 and C8 columns [33-36,46,56,61,68,70,74,100,105,109]. Moreover, reports on small bore columns with internal diameter of 1 mm [96], as well as columns modified with hydrophobic groups have been reported [65]. Columns with polymer-coated stationary phase such as polyvinyl alcohol [38] as well as phenyl groups [81], cyano groups [69] or β-cyclodextrin [110] have been reported. Other example refers to monolithic columns coated with silica in order to assure low pressure in the column and thus increase the efficiency of separation [77]. Separation of test compounds was performed in isocratic conditions [28,31,36,40,45,46,52,57,59,60,63,71,76,77,90,91,99,109] or in gradient [24-26,30,32-34,38,43,44,47,49,53,64,92,107,105,111] with different eluents as mobile phase [23,24,28,30,31,40,45-46,52,56,58,59,60,62,64,66-69,74,77,80,87-91,93,96,98-101,106,111-113].

UV detection at wavelength of 215 nm [69], 220 nm [106], 226 nm [60], 228 nm [36,59], 230 nm [35,40,51,99], 235 nm [61], 240 nm [33,56,58,81,111], 242 nm [91,101], 250 nm [109], 254 nm [65,79,92-94,96,97,100,107], 255 nm [67,90] was reported to be appropriate. Furthermore diode-array detector (DAD) [34,52,74,76,80,105,114,115] was also reported to be applicable in benzodiazepine analysis as well as electrochemical detector [109]. High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) [19,20,38,45-49,63,71,72,77,106,112,113,115] has been extensively reported. Moreover, tandem mass spectrometry (HPLC-MS-MS) [21-32,37,43,53,57,64,66,68,76,78,82-85,88,104,108] and HPLC-DAD-MS has also been proposed [38,73].

Ionization methods used in benzodiazepine analysis:

- electrospray ionization in positive mode (ESI +) [23,24,28,29,31,32,37,38,43,44,49,53,57,63,66,71,72,77,82,84,88,106,107]; and electrospray ionization in negative mode (ESI -) [72],
- atmospheric pressure ionization combined with electrospray ionization (API-ESI) [22,48];
- chemical and atmospheric pressure ionization (APCI) [19,20,46,64,77,112];
- thermospray ionization (TSP) [78,113,115];
- sound spray ionization (SSI) [45].

In MS analysis triple quadrupole mass analyzer [21,24,28,30,38,43,82] was frequently utilized. Also single quadrupole [71] and ion trap mass analyzer [49,76] were used. Analysis of thermolabile benzodiazepines may cause problems, therefore tandem mass spectrometry with thermospray ionization was proposed. This technique is applicable for most benzodiazepines, except for ketazolam, where complete decomposition to diazepam was observed when analysis was run in this particular condition [78]. HPLC-MS-MS has also been utilized to detect diazepam as a contamination in herbal remedies and food supplements. The addition diazepam increased the effect of this compound [87]. However, LC-MS method has limitations, *i.e.*, ionization matrix effects. Alteration in response due to the presence of co-eluting compounds that may increase or reduce ionization of the analyte [116].

Among other techniques for the identification of benzodiazepine derivatives in the pharmaceutical preparations, micellar liquid chromatography (MLC) has been utilized [117,118]. MLC was used for the determination of the anticonvulsant drugs such as chlordiazepoxide and diazepam in capsules, pills, tablets, infusions, drops and suppositories. MLC was

also utilized for estimation of bentazepam, halazepam, oxazepam, pinazepam and tetrazepam in pills and capsules.

Honeychurch *et al*. has determinated flunitrazepam and nitrazepam by high-performance liquid chromatography dual electrode detection (LC-DED) in the reductive-reductive mode, using a carbon fibre veil electrode (CFVE) in conjugation with a glassy carbon electrode. They obtained a simple and convenient method to identify both benzodiazepines in liquid samples with a detection limit of 20 ng mL^{-1} [119]. Electrochemical methods in the analysis of drugs are constantly being improved. De Carvalho *et al*. used ion-pair chromatography with pulsed amperometric detection for evaluation of various diuretics from pharmaceutical formulation [120].

Ultra-Performance Liquid Chromatography (UPLC) may also be applied in determination of benzodiazepines. This utility can be exploited for screening, tranquilizers identification and antidepressant drugs determination in pharmaceuticals. Applying this technique, one can determine therapeutic concentration and toxic dose of benzodiazepines in blood sample [16,121]. A precise, UPLC-TOF-MS screening method for searching illicit drugs and medicines was performed by Pedersen *et al*. They have indicated the most commonly prescribed benzodiazepines in about one thousand blood samples, from traffic cases victims. Limit of identification (LOI) obtained in this survey was ranging from 0.001-0.1 mg kg^{-1} in whole blood samples, which made this method very effective in forensic toxicology. UHPLC-MS/MS approach has been used to determine the benzodiazepines level in case of brain death diagnosis (BDD) [122].

3.2. Thin-layer chromatography

Thin-layer chromatography (TLC) has been used for the determination of several benzodiazepines (diazepam, nitrazepam, flunitrazepam, chlordiazepoxide, clonazepam, tetrazepam, oxazepam and temazepam) in pharmaceutical formulations such as tablets, infusion solutions or suspension [10,123]. TLC technique has also been used for the determination of benzodiazepines in biological material [124,125], primarily in serum [18,126,127], plasma [128], whole blood [129-131], urine [128,130-135], and less commonly in stomach content [128]. TLC also allows detecting metabolites in analyzed material [18,124-128,131,133,135]. Liquid-liquid extraction and solid-liquid extraction was described to be the most commonly used extraction methods in pharmaceutical specimens or biological samples [128]. The extraction solvents commonly used for benzodiazepine extraction were: benzene [18]; ethyl ether [10,126,128]; methanol [10]; acetone [10]; chloroform [10]; dichloroethane [128]; toluene [128]; and a solvent mixture composed of: chloroform: isopropanol (3:1) [133]; benzene: dichloromethane (4:1) [135]; chloroform: ethanol (5:1) [10]; toluene: heptane (4:1) [128].

Extraction of test compounds from biological samples followed by purification of analytes is usually performed by acidic hydrolysis, which in consequence leads to formation of the easily determined benzophenones. Unfortunately, it is impossible to transform alprazolam, triazolam loprazolam to derivatives [132-134,136,137]. Typically, TLC utilizes silica-coated glass plates [123] as well as RP-18 plates coated with a non-polar adsorbent [128], with the addition of fluorescent agent F254 [128,138]. Based on literature, commonly used, mobile phase solvents mixtures for TLC are presented in Table 1.

Schwartz and Carbone [131] used two-dimensional thin-layer chromatography for the detection and determination of medazepam and its metabolites in blood. Diazepam, temazepam, ortetrazepam was analyzed using UV detection at a wavelength of 254 nm, in terms of absorbent-coated plates with the addition of the fluorescent agent F254 [10,128]. Alprazolam was detected at 246 nm, and infrared light has also been used as detection method of analyte spots after called: sulfuric acid (diazepam, oxazepam and chlordiazepoxide) [18,126]; Bratton-Marshall reagent (flurazepam and halazepam and their metabolites as aminochlorobenzofenones) [128,134]; stannous chloride, ammonium chloride, sodium nitrite and Griess reagent (oxazepam and nitrazepam) [128]; red fuming nitric acid (chlordiazepoxide) [124]; sulfuric acid, sodium nitrite solution, ammonium sulfamate solution and an ethanolic solution of the dihydrochloride N-(1-naphthyl) ethylenediamine (clonazepam, nitrazepam) [10].

The quantitative analysis of selected benzodiazepine derivatives was performed by densitometry scanning with appropriate wavelengths (*e.g.* diazepam and oxazepam at 380 nm, and chlordiazepoxide at 390 nm) [18,20,124,126,133,135]. In case of a lack of standards, the identification was performed by TLC coupled with MS techniques [139,140]. TLC technique is also hyphened with mild ionization method, known as liquid secondary ion mass spectrometry (LSIMS) to determine rilmazafone and its three metabolites. TLC-FAB-MS technique has also been used to analyze midazolam in plasma [139,140]. TLC method allowed detection of benzodiazepines even at nanogram concentration (identified only 5 ng mL^{-1} 2-amino-5-nitrobenzophenone in the sample of urine or the same amount of oxazepam in serum) [18,126,132,133,135,141].

Table 1. Solvents mixtures composition for mobile phase separation in TLC.

Solvents mixtures composition for mobile phase separation				
Two-component systems	**Ternary systems**	**Quaternary systems**	**Five components systems**	**Single solvents**
• diethyl ether: dioxane (40:60) [126] • chloroform: acetone (90:10) [119] • ethyl acetate: hexane (1:1) [10] • benzene: ethanol (5:1) [10] • nitromethane: ethyl acetate (17:3) [10] • ethyl acetate: heptane (3:1) [10] • chloroform: methanol (10:1) [10] • chloroform: methanol (100:6) [20] • hexane: acetone (3:1) [124]	• chloroform: methyl acetate: methanol (70:25:5) [135] • dichloretan: methanol: water (95:5:0.2) [135] • chloroform: i-propanol: acetic acid (17:2:1) [135] • diethyl ether: 25% ammonia: benzene (80:10:10) [126] • hexane: diethyl ether: acetic acid (80:10:10) [129] • cyclohexane: chloroform: dietylamina (50:40:1) [124] • toluene: ethyl ether: methanol (15:20:0.7) [135] • chloroform: methanol: diethyl ether (85:15:10) [135] • benzene: i-propanol: 25% ammonia (85:15:1) [135] • butanol: chloroform: 25% ammonia (50:50:1) [135] • benzene: n-propanol: 28% ammonia (90:30:1) [136]	• cyclohexane: chloroform: methanol: ammonium hydroxide (50:40:12:1) [125]	benzene: dioxane: 25% ammonia: methanol: ethanol (60:25:5:10:10) [125]	Toluene, ethyl acetate [10,124].

HPTLC equipped with auto-sampler and densitometry scanner, is a sensitive and appropriate method for qualitative and quantitative analysis in pharmaceutical, toxicological, environmental, forensics and food testing [141,142]. HPTLC has been used for the determination of benzodiazepines in simple and complex pharmaceutical specimen, and for the contaminants detection in different dosages. Clotiazepam, after extraction from tablets, as a pure substance, was identified by densitometry at a wavelength of 243 nm [143]. Alprazolam with sertraline in combination tablet was separated on silica gel plates with the addition of the fluorescent agent by using a solvent mixture - carbon tetrachloride: methanol: acetone: ammonia (12:3:5:0.1). Detection was performed densitometrically at 254 nm [144,145]. Clonazepam in tablets with escitalopram oxalate combination, as well as other drugs, were identified on plates coated with silica gel 60 F254, using toluene: ethyl acetate: triethylamine (7:3.5:3) as the mobile phase at 258 nm. This allowed for a highly sensitive analytical method [108]. HPTLC was also reported to detect contaminants in pharmaceutical specimen by HPTLC-UV/FTIR and identify flurazepam hydrochloride and its contaminants in the capsules and powder mass. FT-IR detection increased sensitivity and rate of analysis. Spectrophotometer equipped with DRIFT adapter allowed powder analysis without tableting, which is important for the samples which changing their properties during compression. The utility of a new type of adsorbent with the addition of magnesium tungstenate characterized by blue fluorescence light at 254 nm was also reported. Solvent mixture composed of toluene: methanol: ethyl acetate: ammonia (8:1:1:0.1), and densitometric measurements were performed at 230 nm [146]. Stahlmann and Kovar [147] used HPTLC-UV/FTIR in combination with the DRIFT to detect contaminants in tablets and chlordiazepoxide powder mass. HPTLC method was suitable for the identification of benzodiazepine derivatives in

biological fluids such as blood serum or plasma [128,143,148,149]. Clotiazepam was determined by fluorodensytometric method in plasma at 313 nm [143] as well as midazolam in serum determined by HPTLC coupled with FAB-MS [128]. In order to develop a rapid and sensitive method for benzodiazepines in the serum, prior sample extraction using silica gel-coated plates were used. Three systems were applied - cyclohexane: toluene: dietylamine (75:15:10), chloroform: methanol (90: 10), and chloroform: acetone (80:20). To visualize spots of separated compounds, the plates were treated with chemical reagents and then subjected to UV at 254 and 366 nm [149]. HPTLC was also used in forensic analysis to determine benzodiazepine derivatives in food products such as soft drinks, fruit juices, biscuits. Diazepam was detected by densitometry at 230 nm or coupled with MS [150,151]. For benzodiazepines separation in HPTLC technique plates coated with silica gel have been commonly used. The analysis of eighteen benzodiazepines, first appropriately prepared, was also conducted on plates with dihydroxy groups (OH), cyano (CN) and an amino (NH_2). Mobile phase constituted of a mixture of carbon tetrachloride: ethyl acetate, and water: methanol - used in different proportions. Separation in reverse-phase on plates with active NH_2 groups was proved to be unsuitable for most of studied compounds, with the exception of chlordiazepoxide and medazepam. It has been shown that these plates can be used for separation of benzodiazepines, not only in normal phase, but also in reversed-phase, depending on the properties of the mobile phase [136,151]. In a few cases the TLC plate was stored in methanol before analysis, and then dried at about 110°C [108,146, 147].

3.3. Gas chromatography

Gas chromatography (GC) has also been used in the analysis of benzodiazepine derivatives extracted from urine [152-158] or blood [158-162] using for example n-butyl chloride [154] or a mixture of chloroform-isopropanol (9:1) [156]. Derivatization process is required to convert the compounds into number of volatile derivatives that could be analyzed in gaseous phase. In some cases, the derivatization process was not necessary, for example: during the chromatographic injection, oxazepam undergoes thermal degradation and was determined by its decomposition products. On the other hand, direct gas chromatography method was used to avoid derivatization procedure [157,162]. Detection of selected compounds was carried out using an electron capture detector (ECD) [159,162]. Gas chromatography coupled with mass spectrometry (GC-MS) or GC-MS-MS with electron impact ionization (EI) [155] positive chemical ionization (PCI) and negative chemical ionization (NCI) [155,160,161], were used for more precise analysis [153-156,158-161,163,164].

3.4. Spectrophotometric

There are few papers describing UV-Vis detection methods in quantitative determination of benzodiazepines. In some of them, the spectrophotometric determination of chlordiazepoxide was reported [165-167]. Spectrophotometric method was also used to determine midazolam in different pharmaceutical forms [142,168], diazepam in tablets [169] and other pharmaceuticals available on the market [170]. Benzodiazepines such as diazepam, clonazepam bromazepamu were assayed by spectrophotometric methods in drugs and urine samples [171]; and flurazepam in urine and blood [172]. Another method for spectrophotometric determination of bromazepam and its metabolites in urine should be performed utilizing hydrolysis reaction of the compounds before analysis [173]. UV-spectrophotometry of ruthenium ion (III) was used to determine estazolam and triazolam [174]. UV spectrophotometry is also applied for the quantitative analysis and identification of nitrazepam content and its metabolites in pharmaceuticals, urine and blood [175]. It is also applicable in the determination of nitrazepam in medicines and urine samples [176].

So far, there were several articles describing the utility of direct derivative UV-spectrophotometry in the analysis of benzodiazepines [142]. This method was used to determine nitrazepam, and clonazepam in biological fluids, and found that the use of the fifth derivative spectrum for nitrazepam and sixth derivative spectrum for clonazepam allow to determine these compounds directly in urine. Moreover, use of a fourth-order derivative of spectrum provides the determination of these compounds in the plasma [177]. The method of the fourth-derivative spectrum was also used to determine diazepam and oxazepam [178] considering kinetics of prazepam degradation in aqueous solutions [179]. In turn, the secondary-derivative spectrum was used for the determination of diazepam in tablet and the mixture of imipramine hydrochloride [180]. First-order derivative method was employed to determine chlordiazepoxide [181] and oxazepam [182] in pharmaceutical formulations. Another technique used for determination of the amount of benzodiazepines was difference spectrophotometry in the UV-VIS. It was used for determination of chlordiazepoxide, demoxepam [183], and nitrozepam in tablets [184]. Direct spectrophotometric assay was used to eliminate products of degradation in prazepam's and nitrazepam's analysis [185]. This method was also

used in chlordiazepoxide and diazepam determination [186]. The spectrofluorimetry was also proposed as a method for quantitative analysis of benzodiazepines. It was used for the determination of nitrazepam and oxazepam in pharmaceuticals [187], flurazepam and its major metabolites in blood and urine [172], and diazepam, clonazepam, bromazepamu, lorazepam in drugs as well as biological fluids [171,188]. Fluorimetric method has also been proposed for the quantification of benzodiazepine derivatives containing hydroxyl group at position 3 (oxazepam, lorazepam, cinolazepam, temazepam) in urine and serum [189]. Fluorimetric detection using flow - injection technique has also been used in the analysis of oxazepam, diazepam and nitrazepam in pharmaceuticals, after prior hydrolysis of these compounds [190].

3.5. Capillary electrophoresis

Capillary Electrophoresisis involves many different electromigration techniques. Capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE), capillary isotachophoresis (CITP), micellar electrokinetic capillary chromathography (MECC) and capillary electrochromatography (CEC) are the most popular examples of methods used in analysis of biological samples [191]. Also, CE is a method of choice in drugs analysis according to the European Pharmacopoeia 5th edition [192].

Capillary electrophoresis used a magnetic field to give the flow to electrically charged particles; the whole procedure is usually carried in liquid medium. Charged particles move in fluid with various speed and direction, which enables their separation . The consequence of this mechanism is the possibility of separation polar ion compound, polar non-ion compounds, non-polar non-ion compounds, compounds with considerable molecular weight and chiral particles [191].

Benzodiazepines have very similar hydrophobicity, which make them difficult to analyze by CZE, so different approach has been proposed. Kapnissi *et al.* used capillary electrochromatography (CEC) to separate oxazepam, lorazepam, nitrazepm, flunitrazepam, temazepam and diazepam from sample [193]. A few publications are devoted to capillary electrophoresis as a method of analysis of benzodiazepines. However, Blas *et al.* found CEC-MS(TOF) as a effective manner to separate benzodiazepines from urine sample. These drugs have been quantified to 1 ng mL^{-1} in urine [194]. The fast separation of seven toxic drugs, including benzodiazepines, was performed by Ohyama. The total time of whole separation took less than 9 minutes [195].

Hancu *et al.* used MECC to determinate 8 benzodiazepines and for the study of their stability in acidic medium. They obtained a reliable and fast method with a separation time below 20 minutes [196]. Suzuki *et al*. used dextran sulfate and SDS as running buffer to obtain better separation of benzodiazepines by means of MECC [197]. Optimized CE-MS was used by McLean *et al*. to lower LOD of benzodiazepines, improve migration time and correlation coefficient of calibration plots [198].

3.6. Electrochemical methods

HPLC, CZE, spectrophotometry are methods which are most used in pharmaceutical formulation analysis. All of them are precise and sensitive techniques but, unfortunately, require a complicated sample preparation and the use of appropriate detectors. When the investigated drug has a very low absorption in the UV region, these method, could be elusive. Therefore, some authors reported the use of electrochemical method in pharmaceutical analysis. A simple, accurate and inexpensive electrochemical method was proposed by El Shal. He used glassy carbon electrode (GCE), which had good electrical conductivity and wide potential range. The analysis was performed in different buffer solution with various pH. He obtained rapid, fast method avoiding sample preparation step for the determination of benzodiazepines and other drugs in biological sample and pharmaceuticals [199]. Electrochemical behavior of benzodiazepines could be used for their determination by mercury electrode. Direct square-wave voltammetry (SWV) and square-wave cathodic stripping voltammetry (SWCSV) was used to determine clonazepam, bromazepam, midazolam, diazepam, medazepam, and flurazepam over a wide range of concentrations [200]. Dos Santos *et al.* and Puzanowska *et al.* used an adsorptive cathodic stripping voltammetry (AdCSV) for selective determination 1,4-benzodiazepines from pharmaceuticals [201,202]. Dos Santos employed hanging mercury drop electrode as a working electrode and a Ringer buffer as a supporting electrolyte. Application of solid state voltammetric methods for drugs identification is a relatively new approach. Doménech-Carbó used voltammetry of microparticles (VMP), which provided analytical information on the composition and structure of sparingly soluble materials. This approach was employed for screening adulterants in herbal drugs. One of the most important advantages, of this kind of analysis is the possibility of analyzing very low amounts of sample and avoiding sample preparation step. He used the same method for screening 1,4-benzodiazepines in slimming herbal formulation [203].

3.7. Immunoenzymatic assay

Screening assays for elicit and most commonly drugs are widely available. Immunological methods are methods of choice, when we need to examined a few samples in short time. One of them is: radioimmunoassay method (RIA), enzyme immunoassay (EIA), immunofluorescence (FPIA) and based on the inhibition of latex agglutination (LAI). Nevertheless, there is some insufficient sensitivity to detect usually prescribed benzodiazepines. Attempts to improve immunoenzymatic methods have been reported. Beck *et al.* modified EMIT (enzyme-multipled immuno technique) and FPIA (fluorescence polarization immunoassay) systems to obtain reliable tests for oxazepam and other benzodiazepines. They also compared these tests with GC-MS [204]. Benzodiazepines are notably problematic in screening because of their rapid elimination and high threshold of sensitivity [205]. Boussairi has attempted to determine benzodiazepines in urine samples by immunoezymatic method. His research group demonstrated the benzodiazepines in half of previously marked as negative urine samples [206]. Other authors investigated adinazolam and its major metabolites by FPIA and confirmed it by HPLC. As a result, both assays are acceptable for detecting the presence of adinazolam in human urine [207]. Immuoenzymatic assay allowed determining benzodiazepines not only in urine samples, but also in blood and serum, with acceptable sensitivity [208,209]. Although immunoassays allow rapid detection of commonly accepted benzodiazepines; it is advisable to use the reference methods such as HPLC, GC or CE.

4. Conclusions

In this article the information concerning the current available analytical methods for the determination of BDZs in biological materials and pharmaceutical formulations were presented. Methods reported in this paper allowed for a simple, accurate, rapid and reproducible quantification of BDZs in pharmaceutical formulations as well as biological samples. The described approaches might be very useful for pharmaceutical laboratory and researches in which extensive benzodiazepines analyses are performed.

References

[1] M. Gerecke, Br. J. Clin. Pharmacol. 16, 11 (1983)
[2] O.J. Bogunovic, S.F. Greenfield, Psychiatr. Serv. 55, 233 (2004)
[3] E. Shorter, Benzodiazepines. A Historical Dictionary of Psychiatry (University Press, Oxford, 2005) 41–42
[4] K.T. Olkkola, J. Ahonen, Handb. Exp. Pharmacol. 182, 335 (2008)
[5] M. Lader, Expert. Rev. Neurother. 8, 1189 (2008)
[6] J.W. Devlin, R.J. Roberts, Crit. Care. Clin. 25, 431 (2009)
[7] W. Sieghart, J. Psychiatry. Neurosci. 19, 24 (1994)
[8] J.H. Skerritt, G.A.R. Johnston, Eur. J. Pharmacol. 89, 3–4, 193–198 (1983)
[9] A. Zejc, M. Gorczyca, Chemia leków: dla studentów farmacji i farmaceutów (Wydawnictwo Lekarskie PZWL, Warszawa, 2002) (in Polish)
[10] Polish Pharmacopeia VI. (Polskie Towarzystwo Farmaceutyczne, Warszawa, 2002) (in Polish)
[11] L.L. Brunton, J.S. Lazo, K.L. Parker, Farmakologia Goodmana & Gilmana (Wydawnictwo Czelej, Lublin, 2007) (in Polish)
[12] H.P. Rang, M.M. Dale, J.M. Ritter, Farmakologia kliniczna (Wydawnictwo Czelej, Lublin, 2001) (in Polish)
[13] J.C. Stevens, M.H. Pollack, J. Clin. Psychiatry 66, 21 (2005)
[14] J. Riss, J. Cloyd, J. Gates, S. Collins, Acta Neurol. Scand. 118, 69 (2008)
[15] O.H. Drummer, Forensic. Sci. Rev. 14, 1 (2002)
[16] K. Wiese-Simonsen, S. Hermansson, A. Steentoft, K. Linnet, J. Anal. Toxicol. 34, 1 (2010)
[17] K. J. Lee, S. In, H. Choi, H. Chung, J. Chrom. B. 879, 878 (2011)
[18] S.R. Sun, J. Pharm. Sci. 67, 1413 (1978)
[19] A. Miki, M. Tatsuno, M. Katagi, M. Nishikawa, H. Tsuchihashi, J. Anal. Toxicol. 26, 87 (2002)
[20] J. Valentine, R. Middleton, C. Sparks, J Anal. Toxicol. 20, 416 (1996)
[21] O. Quintela, F.L. Sauvage, F. Charvier, J.M. Gaulier, G. Lachâtre, P. Marquet. Clin. Chem. 52, 1346 (2006)
[22] J. Feng, L. Wang, I. Dai, T. Hrmon, J.T. Bernert, J. Anal. Toxicol. 31, 359 (2007)
[23] M. Laloup, M. del Mar Ramirez Fernandez, G. De Boeck, M. Wood, V. Maes, N. Samyn, J. Anal. Toxicol. 29, 616 (2005)
[24] M. Chèze, G. Duffort, M. Deveaux, G. Pépin, Forensic Sci. Int. 153, 3 (2005)
[25] P. Kintz, M. Villain, V. Cirimele, G. Pépin, B. Ludes, Forensic Sci. Int. 145, 131 (2004)
[26] M. Concheiro, M. Villain, S. Bouchet, B. Ludes, M. López-Rivadulla, P. Kintz, Ther. Drug Monit. 27, 565 (2005)

[27] B.E. Smink, M.P. Mathijssen, K.J. Lusthof, J.J. de Gier, A.C. Egberts, D.R. Uges, J. Anal. Toxicol. 30, 478 (2006)
[28] O. Papini, C. Bertucci, S. Pereira da Cunha, N.A. Guinaim dos Santos, V.L. Lanchote, J. Pharm. Biomed. Anal. 40, 389 (2006)
[29] M.R. Fuh., S.W. Lin, L.L. Chen, T.Y. Lin, Talanta 72, 1329 (2007)
[30] M. Chèze, M. Villain, G. Pépin, Forensic Sci. Int. 145, 123 (2004)
[31] C. Wing-Chi, Y. Tsan-Sang, W. Ming-Kei, C. Lai-Ping, M.V. King-Kuen, Forensic Sci. Int. 162, 95 (2006)
[32] M. Laloup, M. del Mar Ramirez Fernandez, M. Wood, V. Maes, G. De Boeck, Y. Vanbeckevoort, N. Samyn, Anal. Bioanal. Chem. 388, 1545 (2007)
[33] M.N. Uddin, V.F. Samanidou, I.N. Papadoyannis, J. Liq. Chrom. Rel. Technol. 31, 1258 (2008)
[34] W. He, N. Parissis, T. Kiratzidis, J. Forensic Sci. 43, 1061 (1998)
[35] G.W. Kunsman, J.E. Manno, M.A. Przekop, B.R. Manno, K.A. Llorens, C.M. Kunsman, J. Chromatogr. 568, 427 (1991)
[36] P.K. Kunicki, J. Chromatogr. 750, 41 (2001)
[37] S. Hegstad, E.L. Øiestad, U. Johansen, A.S. Christophersen, J. Anal. Toxicol. 30, 31 (2006)
[38] X.P. Lee, T. Kumazawa, J. Sato, Y. Shoji, C. Hasegawa, C. Karibe, T. Arinobu, H. Seno, K. Sato, Anal. Chim. Acta 492, 223 (2003)
[39] M. Segura, J. Barbosa, M. Torrens, M. Farre, C. Castillo, J. Segura, R. de la Torre, J. Anal. Toxicol. 25, 130 (2001)
[40] W.M. Wayne, J. Pawliszyn, J. Pharm. Biomed. Anal. 26, 899 (2001)
[41] M. Villain, M. Concheiro, V. Cirimele, P. Kintz, J. Chromatogr. B 825, 72 (2005)
[42] R. Kronstrand, I. Nyström, M. Josefsson, S. Hodgins, J. Anal. Toxicol. 26, 479 (2002)
[43] R.C. Irving, S.J. Dickson, Forensic Sci. Int. 166, 58 (2007)
[44] R.A. Anderson, M.M. Ariffin, P.A.G. Cormack, E.I. Miller, Forensic Sci. Int. 174, 40 (2008)
[45] M. Shimizu, T. Uno, H. Tamura, H. Kanazawa, I. Murakami, K. Sugawara, T. Tateishi, J. Chromatogr. B 847, 275 (2007)
[46] E.R. Lepper, J.K. Hicks, J. Verweij, S. Zhai, W.D. Figg W, A. Sparreboom, J. Chromatogr. B 806, 305 (2004)
[47] A. Allqvist, A. Wennerholm, J.O. Svensson, R.A. Mirghani, J. Chromatogr. B 814, 127 (2005)
[48] A. Allqvist, A. Wennerholm, J.O. Svensson, R.A. Mirghani, J. Chromatogr. B 430, 180 (1988)
[49] J.L.E. Reubsaet, S. Pedersen-Bjergaard, J. Chromatogr. A 1031, 203 (2004)
[50] G. Podilsky, M. Berger-Gryllaki, B. Testa, A. Pannatier, J. Liq. Chrom. Rel. Technol. 31, 878 (2008)
[51] A. Zarghi, M. Jenabi, Boll. Chim. Farm. 140, 455 (2001)
[52] K.K. Akerman, J. Jolkkonen, M. Parviainen, I. Penttilä, Clin. Chem. 42, 1412 (1996)
[53] X.P. Lee, T. Kumazawa, M. Fujishiro, C. Hasegawa, A. Marumo, Y. Shoji, T. Arinobu, H. Seno, K. Sato, J. Pharm. Biomed. Anal. 41, 64 (2006)
[54] S.N. Muchohi, K. Obiero, G.O. Kokwaro, B.R. Ogutu, I.M. Githiga, G. Edwards, C.R. Newton, J. Chromatogr. B 824, 333 (2005)
[55] T. Kondo, D.C. Buss, P.A. Routledge, Ther. Drug Monit. 15, 35 (1993)
[56] M. Miura, T. Ohkubo, K. Sugawara, N. Okuyama, K. Otani, Anal. Sci. 18, 525 (2002)
[57] T.L. Laurito, G.D. Mendes, V. Santagada, G. Caliendo, M.E. de Moraes, G. de Nucci, J. Mass. Spectrom. 39, 168 (2004)
[58] H. Le Solleu, F. Demotes-Mainard, G. Vinçon, B. Bannwarth, J. Pharm. Biomed. Anal. 11, 771 (1993)
[59] M. Rouini, Y.H. Ardakani, L. Hakemi, M. Mokhberi, G. Badri, J. Chromatogr. B 823, 167 (2005)
[60] J. Knapp, P. Bokník, H.G. Gumbinger, B. Linck, H. Lüss, F.U. Müller, W. Schmitz, U. Vahlensieck, J. Neumann, J. Chromatogr. Sci. 37, 145 (1999)
[61] A. Bolner, F. Tagliaro, A. Lomeo, J. Chromatogr. B 750, 177 (2001)
[62] I.F. Bares, F. Pehourcq, C. Jarry, J. Pharm. Biomed. Anal. 36, 865 (2004)
[63] S.N. Muchohi, S.A. Ward, L. Preston, C. Newton, G. Edwards, G.O. Kokwaro, J. Chromatogr. B 821, 1 (2005)
[64] M. Kollroser, C. Schober, J. Pharm. Biomed. Anal. 28, 1173 (2002)
[65] C. Pistos, J.T. Stewart, J. Pharm. Biomed. Anal. 33, 1135 (2003)
[66] H. Zhua, L. Jinwen, J. Pharm. Biomed. Anal. 39, 268 (2005)
[67] R. Mariot, L. Zangirolami, J. Chromatogr. B 677, 190 (1996)
[68] P. Li, H. Han, X. Zhai, W. He, L. Sun, J. Hou, J Chromatogr Sci. 50,108 (2012)
[69] B. Lehmann, R. Boulieu, J. Chromatogr. B 674, 138 (1995)
[70] A. El Mahjoub, C. Staub, J. Chromatogr. B 742, 381 (2000)
[71] P. Valavani, J. Atta-Politou, I. Panderi, J. Mass Spectrom. 40, 516 (2005)
[72] H. Miyaguchi, K. Kuwayama, K. Tsujikawa,

T. Kanamori, Y.T. Iwata, H. Inoue, T. Kishi, Forensic Sci. Int. 157, 57 (2006)
[73] F.E. Dussy, C. Hamberg, T.A. Briellmann, Int. J. Legal. Med. 120, 323 (2006)
[74] A. El Mahjoub, C. Staub. J Pharm Biomed Anal. 23, 447 (2000)
[75] W.M. Mullett, K. Levsen, D. Lubda, J. Pawliszyn, J. Chromatogr. A 963, 325 (2002)
[76] J. Darius, P. Banditt, J. Chromatogr. B 738, 437 (2000)
[77] A. Bugey, S. Rudaz, C. Staub, J. Chromatogr. B 832, 249 (2006)
[78] A.M.A. Verweij, P.J.L. Lipman, P.G.M. Zweipfenning, Forensic. Sci. Int. 54, 67 (1992)
[79] A. Zevzikovas, A. Bertulyte, V. Dirse, L. Ivanauskas, Medicina 39, 30 (2003)
[80] A. Bugey, C. Staub, J. Pharm. Biomed. Anal. 35, 555 (2004)
[81] M.D. Robertson, O.H. Drummer, J. Chromatogr. B 667, 179 (1995)
[82] C. Moore, C. Coulter, K. Crompton, M. Zumwalt, J. Anal. Toxicol. 31, 596 (2007)
[83] G. Ngwa, D. Fritch, K. Blum, G. Newland, J. Anal. Toxicol. 31, 369 (2007)
[84] E.L. Øiestad, U. Johansen, A.S. Christophersen, Clin. Chem. 53, 300 (2007)
[85] P. Kintz, M. Villain, M. Concheiro, V. Cirimele, Forensic Sci. Int. 150, 213 (2005)
[86] S.K. Gupta, E.H. Ellinwood, J. Chromatogr. A 445, 310 (1988)
[87] Q. Liang, J. Qu, G. Luo, Y. Wang, J. Pharm. Biomed. Anal. 40, 305 (2006)
[88] M.E. Abdel-Hamid, D. Sharma, J. Liq. Chrom. Rel. Technol. 27, 641 (2004)
[89] L.J. Elrod, D.M. Shada, V.E. Taylor, J. Pharm. Sci. 70, 793 (1981)
[90] A.E. Almeida, M.L. Ribeiro, J. Liq. Chrom. Rel. Technol. 22, 1759 (1999)
[91] M.S. Aurora Prado, M. Steppe, M.F. Tavares, E.R. Kedor-Hackmanna, M.I. Santoro, J. Pharm. Biomed. Anal. 37, 273 (2005)
[92] C. Ferreyra, C. Ortiz, J. Pharm. Biomed. Anal. 25, 493 (2001)
[93] P. Pérez-Lozano, E. García-Montoya, A. Orriols, M. Miñarro M., J.R. Ticó, J.M. Suñé-Negre, J. Pharm. Biomed. Anal. 34, 979 (2004)
[94] A. Zevzikoviene, A. Zevzikovas, A. Bertulis, Medicina 39, 37 (2003)
[95] C. Mannucci, J. Bertini, A. Cocchini, A. Perico, F. Salvagnini, A. Triolo, J. Pharm. Sci. 82, 367 (1993)
[96] J.C. Spell, J.T. Stewart, J. Pharm. Biomed. Anal. 18, 453 (1998)
[97] S.E. Roberts, M.F. Delaney, J. Chromatogr. 283, 265 (1984)
[98] V.D. Reif, N.J. DeAngelis, J. Pharm. Sci. 72, 1330 (1983)
[99] A.E. Almeida, M.L. Ribeiro, L. Polese, J. Liq. Chrom. Rel. Technol. 23, 1109 (2000)
[100] A.A. Fatmi, E.A. Hickson, J. Pharm. Sci. 77, 87 (1988)
[101] T. Hudecová, V. Bátorová, S. Hatrík, E. Havránek, Ceska Slov. Farm. 53, 228 (2004)
[102] M. Hu, P. He, Y. Chen, G. Carr, J. Guo, N. Ye, J. Chromatogr. A 1129, 47 (2006)
[103] C. Franzelius, K. Besserer, J. Chromatogr. A 613, 162 (1993)
[104] E.I. Miller., F.M. Wylie, J.S. Oliver, J. Anal. Toxicol.30, 441 (2006)
[105] W.E. Lambert, E. Meyer, Y. Xue-Ping, A.P. De Leenheer, J. Anal. Toxicol. 19, 35 (1995)
[106] T. Kumazawa, X.P. Lee, K. Sato, O. Suzuki, Anal. Chim. Acta 492, 49 (2003)
[107] P. Proença, H. Teixeira, J. Pinheiro, E.P. Marques, D.N. Vieira, Forensic Sci. Int. 143, 205 (2004)
[108] N. Dhavale, S. Gandhi, S. Sabnis, K. Bothara, Chromatographia 67, 487 (2008)
[109] M. Wilhelm, H.J. Battista, D. Obendorf, J. Chromatogr. A 897, 215 (2000)
[110] A. Fedurcova, P. Majek, J. Lehotay, J. Cizmarik, J. Liq. Chrom. Rel. Technol. 29, 2229 (2006)
[111] S. Diallo, E. Bugni, F. Senhadj-Raoul, S. Gasdeblay, D. Marot, M.C. Dessalles, G. Mahuzier, Talanta 55, 721 (2001)
[112] H. Kanazawa, Y. Konishi, Y. Matsushima, T. Takahashi, J. Chromatogr. A 797, 227 (1998)
[113] M. Yoshida, T. Watabiki, T. Tokiyasu, I. Saito, N. Ishida, Nihon Hoigaku Zasshi 47, 220 (1993)
[114] S.M. Sultan, A.H. El-Mubarak, Talanta 43, 569 (1996)
[115] I.S. Lurie, D.A. Cooper, R.F.X. Klein, J. Chromatogr. A 598, 59 (1992)
[116] F.T. Peters, D. Remane, Anal Bioanal Chem. 403, 2155 (2012)
[117] M.F. Cholbi-Cholbi, J.J. Martınez-Pla, S. Sagrado, R.M. Villanueva-Camanas, M.J. Medina-Hernandez, J. Liq. Chrom. Rel. Technol. 27, 153 (2004)
[118] M. Gil-Agustí, S. Carda-Broch, M.C. García-Alvarez-Coque, J. Esteve-Romero, J. Liq. Chrom. Rel. Technol. 23, 1387 (2000)
[119] K.C. Honeychurch, J. P. Hart, J. Solid State Electrochem. 12, 1317 (2008)
[120] L.M. de Carvalho, C.Viana, A. Moreira, P. do Nascimento, D. Bohrer, M. J. Motta, G. da Silveira, J. Solid State Electrochem. 17,

1601 (2013)
[121] P. Proença, et al., Forensic Sci. Int. 227, 85 (2013)
[122] D. Remane, D. Montenarh, M. Meyer, H. Maurer, Ther. Drug. Monit. 0, 1 (2013)
[123] M. Bakavoli, M. Kaykhaii, J. Pharm. Biomed. Anal. 31, 1185 (2003)
[124] J.L. Straughan, W.F. Cathcart-Rake, D.W. Shoeman, D.L. Azarnoff, J. Chromatogr. B 146, 473 (1978)
[125] S. Ebel, H. Schutz, Z. Rechtsmed. 81, 107 (1978)
[126] S.R. Sun, J. Pharm. Sci. 639 (1978)
[127] N. Wad, H. Rosenmund, E. Hanifl, J. Chromatogr. A 128, 231 (1976)
[128] M.M. Hefnawy, J. Pharm. Biomed. Anal. 27, 661 (2002)
[129] A. Zevzikoviene, A. Zevzikovas, A. Bertulis, Medicina 39, 1100 (2003)
[130] G. Kiliuviene, P. Vainauskas, A. Zevzikovas, V. Dirse, Medicina 38, 550 (2002)
[131] M.A. Schwartz, J.J. Carbone, Biochem. Pharmacol. 19, 343 (1970)
[132] R. Jain, Addict. Behav. 25, 451 (2000)
[133] R. Jain, J. Chromatogr. B 615, 365 (1993)
[134] K.K. Kaistha, R. Tadrus, J. Chromatogr. A 154, 211 (1978)
[135] T. Inoue, T. Niwaguchi, J. Chromatogr. B 339, 163 (1985)
[136] J. Sherma, B. Fried, J. Liq. Chrom. Rel. Technol. 28, 2297 (2005)
[137] O.H. Drummer, J. Chromatogr. B 713, 201 (1998)
[138] A. Maślanka, J. Krzek, J. AOAC Int. 88, 70 (2005)
[139] C. Cimpoiu, T. Hodisan, J. Pharm. Biomed. Anal. 21, 895 (1999)
[140] G.W. Somsen, W. Morden, I.D. Wilson, J. Chromatogr. A. 703, 613 (1995)
[141] P.K. Salo, H. Salomies, K. Harju, R.A. Ketola, T. Kotiaho, J. Yli-Kauhaluoma, R. Kostiainen, J. Am. Soc. Mass. Spectrom. 16, 906 (2005)
[142] M. Koba, K. Koba, T. Bączek, Anal. Lett. 42, 1831 (2009)
[143] M. Busch, W. Ritter, H. Möhrle, Arzneimittel-Forschung 35, 547 (1985)
[144] K. Venkateswarlu, R.K. Venisetty, N.R. Yellu, S. Keshetty, M.G. Pai, J. Chromatogr. Sci. 45, 537 (2007)
[145] S.B. Chepurwar, A. A. Shirkhedkar, S.B. Bari, R.A. Fursule, S.J. Surana, J. Chromatogr. Sci. 45, 531 (2007)
[146] S. Stahlmann, T. Herkert, C. Röseler, I. Rager, K.A. Kovar, Eur. J. Pharm. Sci. 12, 461 (2001)
[147] S. Stahlmann, K. A. Kovar, J. Chromatogr. A 813, 145 (1998)
[148] S. Mennickent, A. Sobarzo, M. Vega, C.G. Godoy, M. de Diego, J. Sep. Sci. 30, 2167 (2007)
[149] K. Otsubo, S. Hironori, F. Koujiro, O. Ryozo, J. Chromatogr. B 669, 408 (1995)
[150] R.K. Sarin, G.P. Sharma, K.M. Varshney, S.N. Rasool, J. Chromatogr. A 822, 332 (1998)
[151] P. Ghosh, M.M. Krishna Reddy, V.B. Ramteke, B. Sashidhar Rao, Anal. Chim. Acta 508, 31 (2004)
[152] D.A. Black, G.D. Clark, V.M. Haver, J.A. Garbin, A.J. Saxon, J. Anal. Toxicol. 18, 185 (1994)
[153] R. Meatherall, J. Anal. Toxicol. 18, 369 (1994)
[154] R.E. West, D.P. Ritz, J. Anal. Toxicol. 17, 114 (1993)
[155] R.L. Fitzgerald, D.A. Rexin, D.A. Herold, J. Anal. Toxicol. 17, 342 (1993)
[156] J.L. Valentine, r. Middleton, C. Sparks, J. Anal. Toxicol. 20, 416 (1996)
[157] S. Kinani, S. Bouchonnet, N. Milan, I. Ricordel, J. Chromatogr. A 1141, 131 (2007)
[158] K. Kudo, Leg. Med. 1, 159 (1999)
[159] J.M. Wilson, P.N. Friel, A.J. Wilensky, V.A. Raisys, Ther. Drug Monit. 1, 387 (1979)
[160] E.R. Cairns, B.R. Dent, J.C. Ouwerkerk, L.J. Porter, J. Anal. Toxicol. 18, 1 (1994)
[161] D. Song, S. Zhang, K. Kohlhof, J. Chromatogr. B 686, 199 (1996)
[162] D. de Carvalho, V.L. Lanchote, Ther. Drug Monit. 13, 55 (1991)
[163] D. Borrey, E. Meyer, W. Lambert, S. Van Calenbergh, C. Van Peteghem, A.P. De Leenheer, J. Chromatogr. A 910, 105 (2001)
[164] K. Arnhard, R. Schmid, U. Kobold, Anal. Bioanal. Chem. 403, 755 (2012)
[165] P. Jatlow, Clin. Chem. 18, 516 (1972)
[166] J.H.J. Riddick, Clin. Biochem. 6, 189 (1973)
[167] A.R. Ossman, A.H. el Hassany, Pharmazie 30, 257 (1975)
[168] L.B. Pfendt, G.V. Popovic, I. Supancic, J. Pharm. Biomed. Anal. 13, 1551 (1995)
[169] C.F. Ferreyra, C.S. Ortiz, Pharm. Biomed. Anal. 29, 811 (2002)
[170] S. Liawruangrath, J. Makchit, B. Liawruangrath, Anal. Sci. 22, 127 (2006)
[171] A.A. Salem, B.N. Barsoum, E.L. Izake, Spectrochim. Acta A Mol. Biomol. Spectrosc. 60, 771 (2004)
[172] J.A. de Silva, N. Strojny, J. Pharm. Sci. 60, 1303 (1971)
[173] J. Lopez Valdeon, M.T. Sevilla Escribano, L. Hernandez, Analyst. 112, 1365 (1987)
[174] N. Gallo, V.D. Bianco, P. Bianco, G. Luisi, Farmaco Prat. 38, 205 (1983)

[175] A.M. Diab, Pharmazie 30, 746 (1975)
[176] G.M. Wassel, A.M. Diab, Pharmazie 28, 790 (1973)
[177] F. Randez-Gil, J.A. Daros, A. Salvador, M. de la Guardia, J. Pharm. Biomed. Anal. 9, 539 (1991)
[178] M.E. Abdel – Hamid, M.A. Abuirjeie, Analyst. 113, 1443 (1988)
[179] H.A. Archontaki, I.E. Panderi, E.E. Gikas, M. Parissi-Poulou, J. Pharm. Biomed. Anal. 17, 739 (1998)
[180] P. Umapathi, P. Parimoo, J. Pharm. Biomed. Anal. 13, 1003 (1995)
[181] M.I. Toral, P. Richter, N. Lara, P. Jaque, C. Soto, M. Saavedra, Int. J. Pharm. 189, 67 (1999)
[182] M.I. Toral, M.A. Munoz, S.L. Orellana, J. AOAC Int. 88, 1173 (2005)
[183] A.G. Davidson, J. Pharm. Sci. 73, 55 (1984)
[184] A.G. Davidson, H.W. Li, J. Pharm. Pharmacol. 41, 63 (1989)
[185] F.A. el – Yazib, M.H. Abdel-Hay, M.A. Korany, Pharmazie 41, 639 (1986)
[186] M. Bedair, M.A. Korany, M.E. Abdel-Hamid, Anlyst. 109, 1423 (1984)
[187] M. Gajewska, G. Glass, D. Slezak, Acta Pol. Pharm. 49, 5 (1992) (in Polish)
[188] J. Rodriguez Procopio, P. Hernandez Hernandez, L. Hernandez Hernandez, Alanyst. 112, 79 (1987)
[189] M.O. Walas, F. Belal, M.E. Metwally, M.M. Hefnazwy, J. Pharm. Biomed. Anal. 12, 1417 (1994)
[190] J. Dolejsova, P. Solich, C.K. Polydorou, M.A. Koupparis, C.E. Efstathiou, J. Pharm. Biomed. Anal. 20, 357 (1999)
[191] Z. Witkiewicz, Podstawy Chromatografii (Wydawnictwo Naukowo Techniczne, Warszawa, 2000) (in Polish)
[192] European Pharmacopeia 5th Edition
[193] C.P. Kapnissi I.M. Warner, J. Chromatogr Sci. 42, 238 (2004)
[194] M. Blas,B.R. McCord, Electrophoresis 29, 292182 (2008)
[195] K. Ohyama, M. Wada, Y. Ohba, O. Fujishita, K. Nakashima, N. Kuroda, Biomed. Chromatogr. 18, 396 (2004)
[196] G. Hancu, J. Biochem. Biophys. Methods. 69, 251 (2007)
[197] Y. Suzuki, H. Arakawa, M. Masako, Biomedical Chromatography 18, 150 (2004)
[198] S. McClean, E. O'Kane, W. Smyth, Electrophoresis 21, 1381 (2000)
[199] M. A El-Shal Adv. Pharm. Bull. 3, 339 (2013)
[200] M.M Dos Santos, V. Famila, M.L. Gonçalves, Anal. Bioanal. Chem. 374, 1074 (2002)
[201] M.M. Dos Santos, V. Famila, M.L. Gonçalves, Anal. Biochem. 303, 111 (2002)
[202] H. Puzanowska-Tarasiewicz, W. Misiuk, K. Mielech-Łukasiewicz, L.Kuźmicka, Indian J. Pharm. Sci. 71, 8 (2009) (in Polish)
[203] A. Domenech-Carbo,, M. Martini, L.M. de Carvalho, C. Viana, M. Domenech-Carbo, M. Silva, J. Pharm. Biomed. Anal. 74, 194 (2013)
[204] O. Beck, P. Lafolie, P. Hjemdahl, S. Borg, G. Odelius, P. Wlrbmg Clin.Chem. 38, 271 (1992)
[205] R. Meatherall, A.D. Fraser, Ther Drug Monit. 20, 673 (1998)
[206] A. Boussairi, J.P. Dupeyron, B. Hernandez, D. Delaitre, L. Beugnet, P. Espinoza, O. Diamant-Berger, J. Toxicol. Clin. Toxicol. 34,721 (1996)
[207] A.D. Fraser, A.F. Isner, W. Bryan, J. Anal. Toxicol. 17,427 (1993)
[208] W. Huang, D.E. Moody, J. Anal. Toxicol. 19, 333 (1995)
[209] W. Huang, D.E. Moody, D.M. Andrenyak, D.E. Rollins, J Anal Toxicol. 17, 365 (1993)