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Determination of flumazenil in serum by liquid chromatography-mass spectrometry: Application to kinetics study in acute diazepam overdose

Određivanje flumazenila u serumu tečnom hromatografijom sa masenom spektrometrijom: primena u kinetičkoj studiji kod akutnog predoziranja diazepamom

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Abstract

Backgound/Aim. Flumazenil is benzodiazepine receptor antagonist. It has been studied for a various indications, including reversal of sedation after surgery or diagnostic procedures, awakening of comatose patients in benzodiazepine overdose, or for symptomatic treatment of hepatic encephalopathy. Some drugs, like theophylline, may prolong its elimination half-life. Considering the long half-life of diazepam and its metabolites, concomitant use of theophylline may reduce the need for repeated dosing of flumazenil in patients with acute diazepam poisoning. The aim of this study was to introduce a reliable and accurate method for determining the concentration of flumazenil after therapeutic application in patients with acute poisoning, and using that method to assess whether the kinetics of flumazenil change in the presence of aminophylline (combination of theophylline and ethylenediamine in a 2 : 1 ratio) applied as concomitant therapy. **Methods.** Blood samples from patients with acute diazepam poisoning that received flumazenil at the dose of 0.5 mg, or the same dose with 3 mg/kg of body weight of aminophylline, were collected 1, 3, 10, 30, 60, 120 and 240 min after its intravenous administration. Samples were prepared by solid-phase extraction on Oasis HLB cartridges with ethylacetate as extracting agens. Flumazenil was determined by liquid chromatography with mass spectrometry (LC-MS) in single ion monitoring mode at m/z 304. Separation of flumazenil from matrix compound was performed on Lichrospher RP-8 column using the mixture of acidic acetonitrile and 20 mM of ammonium acetate in water (55 : 45) as a mobile phase. **Results.** The applied analitycal method showed excellent recovery (94.65%). The obtained extracts were much cleaner than the extracts obtained by the same extractant in the process of liquid-liquid extraction. The limit of detection of the LC-MS method described in this paper was 0.5 ng/mL and the limit of quantitation was 1 ng/mL. In the patients treated with both flumazenil and aminophylline, the elimination constant for flumazenil was significantly lower and the elimination half-life was longer ($p < 0.05$) in comparison with the same parameters in the patients who received flumazenil alone. **Conclusion.** The applied LC-MS method for the determination of flumazenil in serum samples of patients with acute diazepam poisoning is rapid, sensitive, precise and specific. Concomitant use with theophylline significantly prolonged elimination of flumazenil during the treatment of acute poisonings with diazepam.

Key words:
diazepam; poisoning; flumazenil; aminophylline; chromatography, liquid; mass spectrometry.

Apstrakt

Uvod/Cilj. Flumazenil je antagonist benzodiazepinskih receptora čiji su efekti ispitivani kod različitih indikacija kao što su reverzija sedacije posle hirurških intervencija ili dijagnostičkih procedura, terapija kome u akutnim trovanjima ili simptomatska terapija hepatične encefalopatije. Pojedini lekovi, kao što je teofilin, mogu dovesti do produženja poluvremena eliminacije flumazenila. Imajući u vidu dugo poluvreme eliminacije diazepama i njegovih metabolita, istovremena upotreba teofilina sa flumazenilom bi smanjila potrebu za ponovljenim davanjem flumazenila kod bolesnika sa akutnim trovanjem diazepamom. Stoga, cilj ovog rada bio je uvođenje pouzdane i precizne metode za određivanje koncentracije flumazenila u krvi nakon terapijske primene kod bolesnika sa akutnim trovanjem, a zatim, primenom ove metode utvrđivanje da li dolazi do izmena u kinetici flumazenila u prisustvu istovremeno primenjivanog aminofilina (kombinacija teofilina i etilendiamina u odnosu 2 : 1). **Metode.** Uzorci krvi bolesnika sa akutnim trovanjem diazepamom koji su dobili samo flumazenil u dozi od 0,5 mg ili istovremeno sa 3 mg/kg aminofilina, uzeti su 1, 3, 10, 30, 60, 120 i 240 min nakon njegove intravenske primene. Uzorci su pripremani čvrsto-faznom ekstrakcijom

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(SPE) na Oasis HLB kertridžima sa etilacetatom kao ekstrakcionim agensom. Flumazenil je određen tečnom hromatografijom sa masenom spektrometrijom (LC-MS) u *single ion monitoring* (SIM) modu na m/z 304. Razdvajanje flumazenila od komponenti matriksa izvršeno je na Lichrospher RP-8 koloni uz korišćenje smeše kiselog acetonitrila i 20 mM amonijum acetata u vodi (55 : 45) kao mobilne faze. **Rezultati.** Primenjena analitička metoda pokazala je odličan analitički prinos (94.65%). Dobijeni ekstrakti bili su čistiji nego ekstrakti dobijeni pomoću istog ekstraktanta nakon tečno-tečne ekstrakcije. Limit detekcije i limit kvantifikacije (LoQ) opisane LC-MS metode bili su 0,5 ng /mL i 1 ng/mL. Kod bolesnika lečenih istovremenom primenom flumazenila i aminofilina, konstanta eliminacije za flumazenil bila je značajno veća, a poluvreme eliminacije značajno duže u odnosu na ove parametre praćene u grupi bolesnika koja je primila samo flumazenil ($p < 0,05$). **Zaključak.** Primenjena LC-MS metoda za određivanje flumazenila u serumu bolesnika sa akutnim trovanjem diazepamom je brza, osetljiva, precizna i specifična. Istovremena primena teofilina značajno produžava eliminaciju flumazenila prilikom lečenja akutnih trovanja diazepamom.

Ključne reči:
diazepam; trovanje; flumazenil; aminofilin; hromatografija, tečna; spektrometrija mase.

Introduction

Flumazenil, an imidazobenzodiazepine, is a competitive antagonist of benzodiazepine receptors. It selectively binds to these receptors in the central nervous system, thus blocking activation of inhibitory gama-aminobutyric acid (GABA)-ergic synapses. This way, flumazenil antagonizes central effects of substances which manifest their activity through benzodiazepine receptors [1–3]. Flumazenil has been studied for a various indications, including reversal of sedation after short-lasting surgery or diagnostic procedures like endoscopy, awakening of comatose patients in benzodiazepine overdose, or for symptomatic treatment of hepatic encephalopathy [4–7].

Flumazenil may be administered as an antidote in acute poisoning with benzodiazepines [8, 9], but it should not be used in patients with the history of epilepsy or with benzodiazepine intoxication combined with tricyclic antidepressants [10, 11]. Flumazenil may precipitate withdrawal syndrome, cardiovascular effects, or seizures in overdosed benzodiazepine dependent patients [8, 12, 13]. Because of contraindications and adverse effects, flumazenil must be used with caution in poisoning with benzodiazepines. Although it increases the level of consciousness in benzodiazepines poisonings, because many benzodiazepines have a longer half-life than flumazenil, resedation is possible soon after application, and therefore, sometimes it is necessary to apply several doses of the drug to improve the therapeutic efficiency [8].

Flumazenil does not alter the pharmacokinetics of benzodiazepines [14], end the extent to which flumazenil antagonizes effects of benzodiazepines depends on the dose and the concentration of both drugs in plasma [1]. The metabolism of flumazenil is rapid and extensive, and takes place in the liver. The medium half-life of flumazenil in plasma is about 54 min (41–79 min) [1], but there are some substances, like theophylline which could prolong its half-life [15].

Determination of flumazenil in serum samples may be carried out using various chromatographic techniques [15–27]. Often it has been applied to high performance liquid chromatography (HPLC) with ultraviolet detection (HPLC-UV) [16–21], but a more specific and sensitive method is liquid chromatography with mass spectrometry (LC-MS) detection [22–25].

Thus, the aim of this study was to introduce a reliable and accurate method for determining the concentration of flumazenil after therapeutic application in patients with acute diazepam poisoning, and using that method to assess whether the kinetics of flumazenil change in the presence of aminophylline combination that contains teophylline and ethylenediamine in a 2 : 1 ratio) applied as parallel therapy, because slowing of elimination may prolong its antidotal action and thus reduce the need for repeated doses.

Methods

Material

Flumazenil and fluoxetine (an internal standard) analytical standards were obtained from the companies Roche (Basel, Switzerland) and Sigma-Aldrich (St. Luis, Missouri, United States), respectively. HPLC grade acetonitrile and methanol, as well as acetic acid, ammonium acetate, ethyl acetate and hydrochloric acid p.a, were obtained from Merck (Darmstadt, Germany). Water was purified by Millipore Milli-Q system. Cartridges for solid-phase extraction Oasis HLB 30 μm, 1 mL, were obtained from Waters (Manchester, United Kingdom).

Blood samples from the two groups of patients (10 persons each) with acute diazepam poisoning, who received flumazenil at the dose of 0.5 mg, or the same dose with the 3 mg/kg of body weight of aminophylline, were collected 1, 3, 10, 30, 60, 120 and 240 min after intravenous administration.

Method

For determination of flumazenil in serum a mass spectrometer with chemical ionization at atmospheric pressure (Finnigan MAT SSQ7000 LC/MS – ESI System) with HPLC P2000 binary pump, degasser SCM1000 and autosampler AS3000 were used. Mobile phase was a mixture of the solution A (acetonitrile: glacial acetic acid = 99 : 1) and B (20 mM of ammonium acetate in water) in the ratio of 55 : 45. The flow rate of mobile phase was 1 mL/min. Separation of flumazenil and internal standard from matrix compound was performed on a column Lichrospher 100 RP-8 E 250-4, 5 μm (Merck), with guard column Lichrochart 4–4 RP-8 (Merck) at ambient temperature after injection of 50 μL of sample.

A mass detector was adjusted to work in a single ion monitoring (SIM) mode for masses m/z 304 and 310 for flumazenil and internal standard, respectively. The electron multiplier voltages was 2,200 V. The capillary and the tube lens voltages were

26.8 V and 115.9 V, respectively. The pressure of the main and the auxiliary gas (N2) was 60 and 150 psi, respectively.

Preparation of a standard solution and samples

The stock standard solution of flumazenil was prepared by dissolving 10 mg in 10 mL acetonitrile and stored at +4ºC. Calibration curve solutions were prepared by adding flumazenil standard solution in pool serum and prepared like serum samples.

Extraction of flumazenil from serum samples was performed on the Oasis HLB cartridge, previously activated with 1 mL of methanol and 1 mL demineralised water. In a serum sample 0.05 mL of internal standard (fluoxetine) and 0.1 mL 1M hydrochloric acid were added. After mixing and centrifugation at 8,360 rpm, a sample was loaded to the activated cartridge. The cartridge was washed with 1 mL of 5% methanol. Elution of flumazenil and the internal standard (IS) is carried out with 3 mL of ethyl acetate. The obtained eluate was evaporated under the stream of air to dryness, reconstituted in 1 mL of mobile phase and analyzed by the LC-MS method.

Comparison of the mean flumazenil maximum concentration (Cmax), elimination constant (Ke) and elimination half-life ($t_{1/2}$) after its applying alone or in combination with aminophylline was done by Student`s *t*-test.

Results

Using the described method, retention times for flumazenil and internal standard were 4.4 min and 2.5 min, respectively. Figure 1 shows the mass spectrum of flumazenil.

Calibration curve solutions were prepared by adding a flumazenil standard solution in pool serum and prepared like serum samples. The calibration curve was linear in the concentrations range of 1; 2.5; 5; 10; 25; 50 and 100 ng/mL (Figure 2).

Chromatograms of the internal standard, serum spiked with

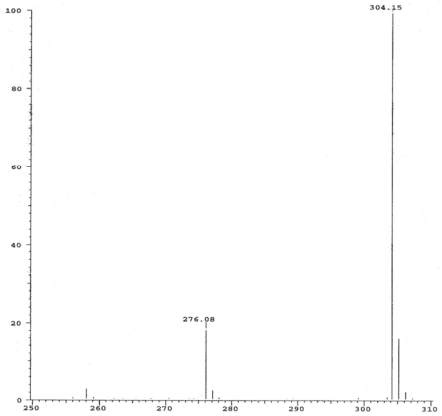


Fig. 1 – Mass spectrum of flumazenil.

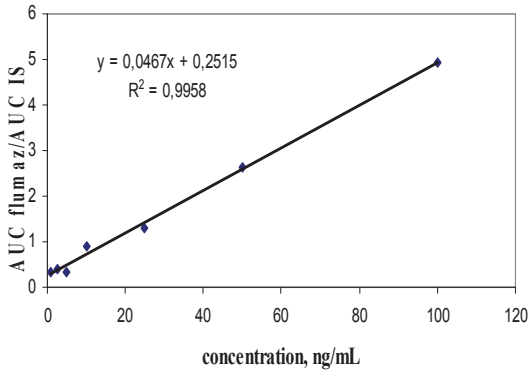
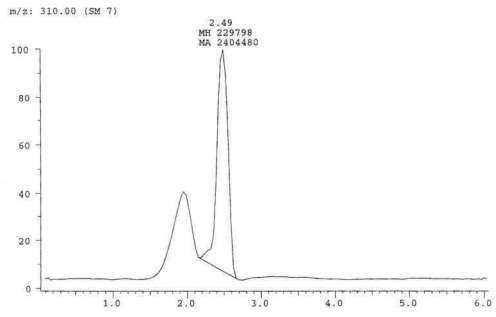
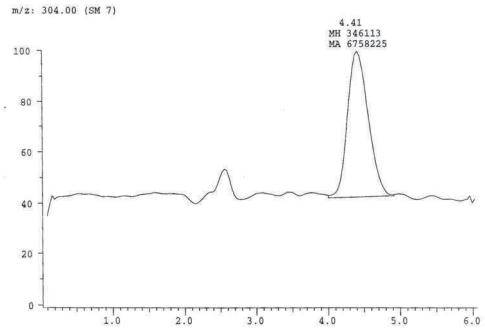


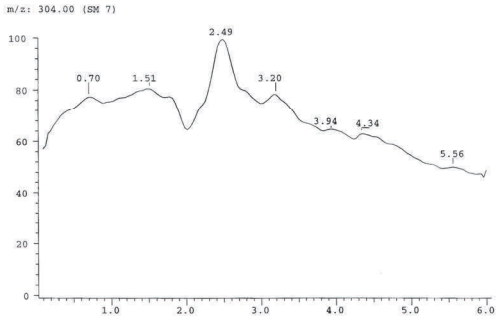
Fig. 2 – Calibration curve of serum spiked with flumazenil. AUC – area under the curve; IS – internal standard



a)



b)



c)

Fig. 3 – Chromatogram of: a) internal standard, b) serum spiked with flumazenil and c) pool serum.

100 ng/mL of flumazenil and pool serum are shown in Figure 3.

The intra-day precision of the method was assessed by calculating the coefficient of variation (CV) for the measured parameter of the method (ratio of peak area of flumazenil and IS) and determined on the same day. It was done by preparing ten flumazenil standard samples with concentration of 10 ng/mL and determining by the LC-MS method. The CV was 5.18%. The inter-day CVs for spiked serum were also acceptable and are shown in Table 1.

The mean analytical recovery was 94.65% (ranged from 91.48 to 99.13%). Table 2 shows the analytically recovery from the serum after solid-phase extraction with ethyl acetate on the Oasis HLB cartridges.

The limit of detection (LoD) was defined as the concentration at which the signal to noise ratio is equal to, or greater than three, and the limit of quantitation (LoQ) was defined as the concentration at which the signal to noise ratio is equal to, or greater than ten. Accordingly, LoD and LoQ were 0.5 ng/L and 1.0 ng/mL, respectively.

Determination of flumazenil in serum samples of patients was carried out on the basis of the equation of the calibration curve, which was obtained upon the analysis of spiked serum. Linear regression of flumazenil was $y = 0.0467 x + 0.2515$ ($R = 0.9958$ for the concentration range of 1 to 100 ng/mL). Main pharmacokinetic parameters of flumazenil including Cmax, Ke and $t_{1/2}$ are listed in Table 3. Student's t-test revealed a significantly lower Ke ($p < 0.05$) and a significantly longer $t_{1/2}$ ($p < 0.05$) in patients treated with both flumazenil and aminophylline.

Table 1

Inter-day coefficient of variation (CV) for determination of flumazenil by liquid chromatography with mass spectrometry (LC-MS) method

Added concentration (mg/L)	1st day			2nd day		
	Obtained concentration (mg/L)	x̄ ± SD	CV	Obtained concentration (mg/L)	x̄ ± SD	CV
1.0	10.15 10.97 9.37	10.16 ± 0.79	7.86%	10.14 8.55 10.09	9.59 ± 0.90	9.42%
10.0	49.23 48.66 52.53	50.14 ± 2.09	4.16%	49.432 48.11 51.98	49.84 ± 1.96	3.94%
100.0	101.23 103.42 98.01	100.88 ± 2.72	2.70%	100.98 102,90 97,16	100.35 ± 2.92	2.91%

x̄ – mean value; SD – standard deviation; CV – coefficent of veriation.

Table 2

Analytical recovery for flumazenil

Flumazenil conc. (ng/mL)	AUC flumazenil/ AUC IS		Recovery (%)
	Standard	Spiked serum	
1	0.3507	0.3208	91.48
2.5	0.4195	0.3909	93.18
5	0.3615	0.3437	95.07
10	0.9528	0.8994	94.39
25	1.3942	1.2920	92.67
50	2.6574	2.6342	99.13
100	5.0906	4.9211	96.67

AUC – arca under the curve; IS – internal standard.

Table 3

Pharmacokinetic parameters of flumazenil in patients treated with flumazenil only or with flumazenil and aminophylline

Patient	flumazenil			flumazenil + aminophylline		
	C_{max} (ng/mL)	Ke (min^{-1})	$t_{1/2}$ (min)	C_{max} (ng/mL)	Ke (min^{-1})	$t_{1/2}$ (min)
1.	34.65	0.0211	32.87	22.78	0.0156	44.46
2.	28.72	0.0168	41.22	92.60	0.0092	75.40
3.	94.33	0.0093	74.62	81.02	0.0050	137.63
4.	76.91	0.0131	52.67	131.87	0.0070	98.84
5.	26.45	0.0100	69.01	28.30	0.0050	137.42
6.	19.82	0.0121	57.29	27.53	0.0075	91.86
7.	41.92	0.0127	54.33	60.56	0.0071	98.07
8.	65.34	0.0091	76.35	21.07	0.0037	188.25
9.	107.27	0.0171	40.46	43.62	0.0097	71.18
10.	23.46	0.0115	60.36	15.42	0.0048	144.96
x̄ ± SD	51.89±31.78	0.0133±0.0039	55.92±14.75	52.48±38.47	0.0075±0.0035	108.81±42.47

C_{max} – maximum concentration; Ke – elimination constant; $t_{1/2}$ – elimination half-life; x̄ – mean value; SD – standard deviation.

Discussion

Isolation of flumazenil from the biological material can be done by liquid-liquid [25] or solid-phase extraction [20]. Previously reported analytical recovery for flumazenil after solid-phase extraction was 78% [21]. In our study, the applied extraction on Oasis HLB cartridges with ethyl acetate as extractant, for preparation of serum samples from acutely poisoned patients showed better recovery (94.65%). Uppon our previous experience in flumazenil determination, the obtained extracts were much cleaner than the extracts obtained by the same extractant in the process of liquid-liquid extraction (data not shown). We found that in comparisson with liquid-liquid extraction, solid-phase exraction is simpler, faster to perform and safer for analyst, which is of great importance when it is necessary to analyze a large number of samples.

The literature describes a variety of chromatographic techniques for the determination of flumazenil such as gas chromatography with nitrogen-phosphorus or mass spectrometric detectors and HPLC-UV, LC-MS [15–27]. Thus, Bun et al. [17] described the HPLC-UV method for determination of flumazenil in serum at 245 nm with the detection limit of 2 ng/mL. Similar result was obtained by Zedkova et al. [18], with the detection limit of 2.5 ng/mL and detection at 250 nm.

Liquid chromatography coupled with mass-spectrometric detection is the most sensitive and the most specific analytical method of drugs in biological samples. Generally, the sensitivity of this method may be increased performing tandem mass spectrometry (LC-MS-MS). The reported limit of detection using LC-ESI-MS method for flumazenil was 2.5 ng/mL[21]. However, the limit of detection of the LC-MS method described in this paper was lower and achieved 0.5 ng/mL, while the limit of quantification was 1 ng /mL.

We applied the described LC-MS method for determination of flumazenil in serum samples and used the obtained data for calculating the pharmacokinetic parameters: c_{max}, $t_{1/2}$ and Ke. According to the literature, the mean c_{max} of flumazenil in plasma after intravenous infusion of 2 mg of this drug was 55 ng/mL [1].

Our data on the flumazenil concentration in serum of patients poisoned by diazepam showed significant inter-individual differences, which are in accordance with the fact that the drug is administered in a fixed dose to patients with different pharmacokinetic properties.

The mean $t_{1/2}$ of flumazenil in the group of patients overdosed with diazepam receiving the drug was 55.92 ± 14.75 which is similar to literature data of 54 min [1]. However, in the group of patients receiving both flumazenil and aminophylline, the mean $t_{1/2}$ of flumazenil was longer (almost double) (108.81 ± 42.47 min). The Ke of flumazenil in this group was also lower (0.0075 ± 0.0035 min^{-1}) than in the group receving only flumazenil (0.0133 ± 0.0039 min^{-1}). Despite the great interindividual variance, the results for the Ke and $t_{1/2}$ of flumazenil showed a statistically significant slowing of flumazenil elimination in the presence of theophylline in blood.

The results of limited previously published studies showed that combined application of flumazenil and theophylline resulted in a prolonged $t_{1/2}$ of flumazenil in rabbits [28]. Also, in patients sedated with midazolam, Bonfiglio et al. [15] revealed that theophylline appeared to significantly prolong the half-life of flumazenil. However, the mechanism of interaction of these two drugs is not known.

The main metabolic transformation of flumazenil involves the activation of carboxylesterase to form flumazenil acid as the major metabolite which is without pharmacological activity. In a small percentage flumazenil may be demehylated through cytochrome P450. The metabolism of theophylline involves mainly hydroxylation and demethylation. In both processes cytochrome P450 oxidase is involved [29,30]. This fact supports the hypothesis that in the case of combined use, flumazenil and theophylline may compete for binding to the same enzyme involved in the process of demethylation.

In recent years, effects of flumazenil and aminophylline have been investigated in reversal of different kinds of anesthesia [31–34]. Concomitant use of both drugs may also be explored, having in mind their synergic action and interactions. Extended half-life of flumazenil in combination with theophylline may also be of importance in the treatment of poisonings with long-acting benzodiazepines.

Conclusion

The applied liquid chromatography with mass spectrometry method for the determination of flumazenil in serum samples of patients acutely poisoned with diazepam is rapid, sensitive, precise and specific. The applied solid-phase extraction gave very good recovery, which is very important considering low concentrations in samples. The method is applicable to the routine determination of flumazenil serum concentrations, as well as in pharmacokinetic studies. Also, our results confirm previous findings that the concomitant use of theophylline significantly prolongs elimination of flumazenil during the treatment of acute poisonings with diazepam.

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