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ORIGINAL ARTICLE

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A specific immunoassay for the detection of flunitrazepam

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Abstract The development of an immunochemical procedure for the determination of flunitrazepam in whole blood is described. Flunitrazepam was derivatized in position 3 of the benzodiazepine ring to a hapten which was coupled to a carrier protein. To obtain antibodies, rabbits were immunized with these immunogens and the collected antisera were tested in a heterogeneous, competitive RIA. The antibodies showed a very specific reaction with flunitrazepam and hardly any cross-reactivity with related 1,4-benzodiazepines. Because of its high specificity the antiserum has the advantage of a definite determination of low levels of flunitrazepam without the risk of false-negative results obtained by using the commercially available group-specific test systems. The drug was extracted from whole blood in a simple batch process with a polystyrene suspension and the extracts were measured by RIA. The advantages of an immunochemical system, such as short analysis time and simple sample preparation, and the exactness of a drug-specific method are combined in this procedure, which allowed the specific and very sensitive determination of flunitrazepam in the low therapeutic range.

Key words Flunitrazepam · Flunitrazepam determination · Radioimmunoassay (RIA)

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Introduction

Flunitrazepam is one of the benzodiazepines, used in low doses as a hypnotic under the trademarks Rohypnol, Fluninoc or Flunitrazepam-ratiopharm. Screening and detection of flunitrazepam in body fluids is necessary, especially in forensic toxicology and traffic medicine. Although it has been in increasing use in the drug scene since 1986 to compensate for withdrawal symptoms [1, 2], its detection is difficult and time consuming, especially in whole blood, because of the low plasma levels (2–15 ng/ml).

The methods available for the determination of flunitrazepam are TLC [3, 4], GC-ECD [5, 6], GC-MS [7, 8], HPLC [9] and immunological methods [10, 11]. The commercially available immunoassays are mostly designed using oxazepam or diazepam as the hapten for antibody production. The application of these assays for the determination of flunitrazepam often leads to false-negative results, because of low cross-reactivity and/or low concentrations in the therapeutic range [12, 13]

The aim of this work was to develop an immunological method with antibodies specific for flunitrazepam. Therefore flunitrazepam was derivatized in the 3-position to preserve the flunitrazepam-characteristic substituents in the positions 1,7,2′ of the benzodiazepine ring and to obtain a hapten with a terminal carboxyl group as coupling position to a carrier protein. Rabbits were immunized with the synthesized immunogens to obtain polyclonal antisera.

Materials and methods

Chemicals

Tetrahydrofuran (THF), *n*-butyllithium (~1.6 mol in hexan), diisopropylamine and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Fluka Chemie AG. Keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) and aerosil were obtained from Pierce. Complete Freund's adjuvant was obtained from Behringwerke, and [*N*-methyl-^{3}H]-flunitrazepam was obtained from Amersham Buchler.

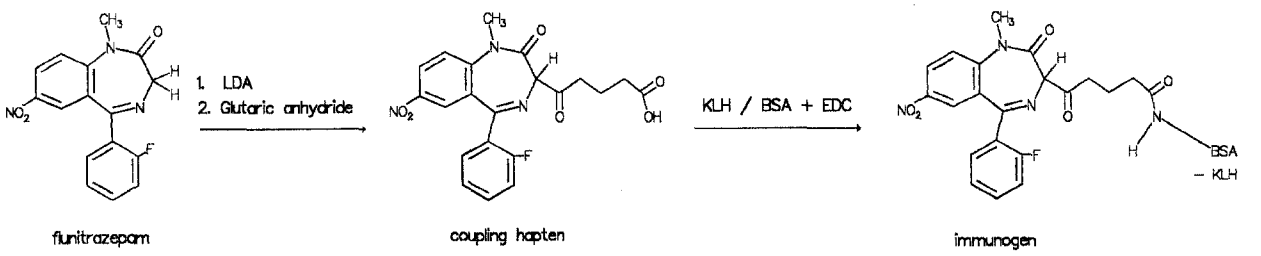

Fig. 1 Synthesis pathway of the immunogen

Thin-layer chromatography

Precoated plates (silica gel 60 GF 254, Merck) were used and detection was achieved by examination under ultraviolet light.

Synthesis of the immunogen

The Li-salt of flunitrazepam was formed by the reaction with lithium diisopropylamine in a low-temperature procedure at –60° C under argon as a protective gas. Lithium diisopropylamine was formed from n-butyllithium and diisopropylamine as described by Reitter et al. [14]. A dry, three-necked flask was equipped with a magnetic stirrer, a thermometer and an argon in- and outlet. The flask was flushed for 10 min with dry argon to remove air. During the following reaction a constant argon flow of 1–2 bubbles per second was maintained through the system. Using a syringe, 3 ml absolute dry THF and 280 µl diisopropylamine (~ 2 mmol) were added and the mixture was cooled in a bath of ethanol and solid CO_2 at –60° C. When the solution reached a temperature of –60° C, 1.5 ml *n*-butyllithium solution was added. The solution was mixed for 15 min at –60° C and then allowed to come to room temperature within 20 min. The lithium diisopropylamine formed was cooled back to –60° C and 313 mg flunitrazepam (~ 1 mmol) in 2 ml absolute THF was added. The reaction could be observed visually because – starting with the first drop of the flunitrazepam solution – the Li-salt is formed and the solution has a deep dark red colour. The mixture was brought to room temperature within 20 min, while the dark red colour turned to brown. The Li-salt of flunitrazepam was cooled back to –20° C and 114 mg glutaric anhydride (~ 1 mmol) was added. The mixture was allowed to come to room temperature and mixed for at least 2 h. The reaction was stopped by adding 10 ml of saturated sodium chloride solution. The solution of the raw product (pH ~ 10) was extracted 4 times with ether. The aqueous solution was brought to pH 6 by adding 1 M-PBS (pH 6). At this pH the flunitrazepam-glutaric-acid derivative formed a precipitate, which was extracted 4 times with methylenechloride and evaporated to dryness. The brown raw product was subjected to thin-layer chromatography. The purification was carried out in the solvent system toluene/diethyl ether/acetic acid/methanol (60/30/9/0.5). The flunitrazepam-glutaric acid derivative was obtained at Rf 0.51, and impurities of flunitrazepam at Rf 0.43. The flunitrazepam-glutaric acid derivative was characterised by ^{1}H- and ^{13}C-NMR.

A C_5-spacer was incorporated in position 3 of the flunitrazepam molecule by reaction with the electrophile glutaric acid anhydride.

For an immunogen to be obtained, the small hapten must be coupled to a carrier protein [15], such as BSA or KLH. KLH has a strong immunogenic effect, because of its large molecular weight (~ 1×10^6 Da). The carbodiimide method is a proven method for coupling carboxyl groups to free amino groups of proteins. The flunitrazepam-glutaric acid derivative reacts first with 1-ethyl-3(3-dimethylamino-propyl)carbodiimide (EDC) to form a reactive intermediate compound, which couples to free amino groups of the protein [16]. The reaction is presented in Fig. 1.

The immunogen was dialysed 3 times overnight against PBS (pH 7.2) to separate free flunitrazepam-derivative and was then lyophilised to get the pure immunogen. To determine the ratio of moles hapten/mol carrier, a defined amount of the immunogen was hydrolysed and the amount of 2-methylamino-5-nitro-2´-fluorbenzophenone (ANFB) formed was analysed by HPLC. Before hydrolysis diazepam was added as an internal standard. In this way, 55 mol hapten per mol KLH and 13 mol hapten per mol BSA could be coupled.

Immunisation and collecting antiserum

The animal experiments were done at the Behringwerke AG in Marburg. The immunogen synthesised in this way was used for immunisation of 3 rabbits with the KLH-conjugate and 2 with the BSA-conjugate. For priming, 750 µg immunogen was dissolved in 1 ml isotonic NaCl solution, mixed with 1 ml complete Freund's adjuvant and the rabbits were injected at 8 subcutaneous sites near to the lymphatic glands. During the following days, the immunogen mixtures were injected intravenously (100 µg of the immunogen in 3 ml of a 0.05% Aerosil suspension). Blood samples were taken 24, 38, 52 and 66 days after priming and at day 67 the animals were sacrificed and the whole blood was collected. Each blood sample was centrifuged to separate the serum, which was stored at –20°C.

Test procedure

A simple immunoelectrophoresis was made initially to show the reaction of the antisera with pure carrier protein in comparison to the reaction with the immunogen (hapten-carrier conjugate). A positive reaction with both could be observed with the first antiserum taken after 24 days. In order to separate the antibodies which recognized flunitrazepam only (as a rule in combination with the carrier) from those which recognized only the carrier-protein, the serum samples were absorbed with the pure carrier protein. After separation of these BSA antibodies in form of a precipitate only the antiserum gained by immunisation with BSA as a carrier showed a positive reaction against the immunogen and no further reaction against the pure carrier. The antiserum gained by immunisation with KLH as a carrier protein showed no reaction in the electrophoresis after this absorption procedure. A reaction with the hapten part was no longer observed for the following tests. Only the antiserum samples produced with BSA as carrier protein were used after absorption with pure BSA. The other antisera were tested without absorption of the antibodies against the carrier part. The immunglobulin fraction of the antisera was isolated from the raw material by a combination of ammonium sulfate precipitation and column chromatography using a DEAE matrix.

A simple RIA-procedure for the determination of the reactivity of the antibodies to flunitrazepam in comparison to those against other benzodiazepines was carried using the procedure described by Dixon [17]. [N-methyl-^{3}H]-flunitrazepam was used as a tracer in a heterogeneous competitive radio-immunoassay and was diluted with PBS to a final activity of 30.000 Cpm in 100 µl. Methanolic stock solutions of various benzodiazepines in a concentration of 1 mg/ml were diluted in the range of 0.01–10000 ng/ml with PBS.

The best antiserum dilution must fulfil the demand that a very low concentration of flunitrazepam in the sample leads to maxi-

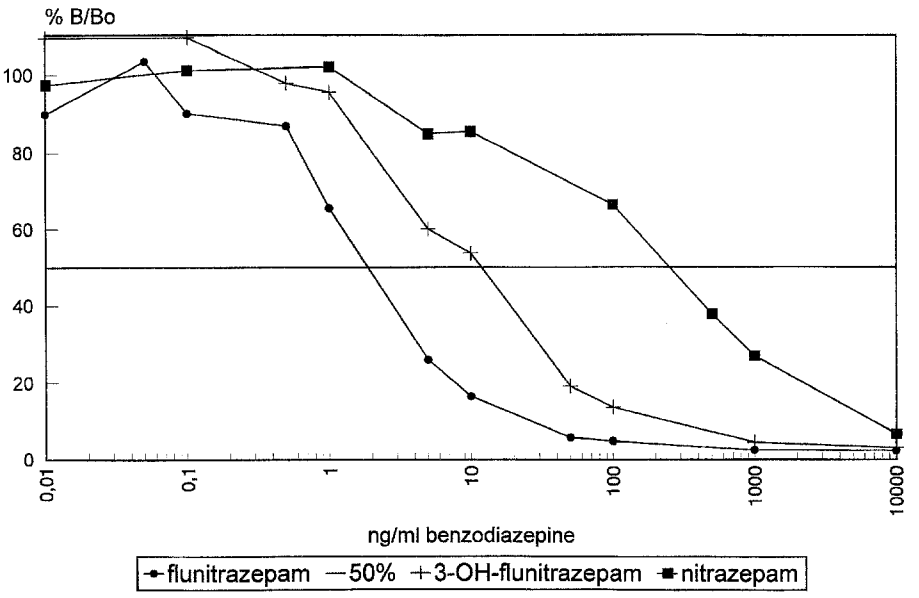

Fig. 2 %B/Bo curves of similar benzodiazepines

Table 1 Cross-reactivity of various benzodiazepines

Benzodiazepine	Cross reactivity
Flunitrazepam	100
3-OH-Flunitrazepam	18
Demethylflunitrazepam	3
Alprazolam	0.9
Nitrazepam	0.8
Diazepam	0.7
Temazepam	0.5
Lorazepam	0.3
Lormetazepam	0.2
Clonazepam	0.05
Aminoflunitrazepam	< 0.02
Demethylaminoflunitrazepam	< 0.02
Oxazepam	< 0.02
Bromazepam	< 0.02

mum reduction of the signal in comparison to the blank. The best results were obtained by using an antiserum dilution of 1:500 for the samples obtained with BSA as carrier and a dilution of 1:250 for the samples with KLH as carrier. For optimizing the test procedure 100 µl of the benzodiazepine dilution and 100 µl of the ^{3}H-flunitrazepam dilution were mixed in a tube and 100 µl PBS was used as a blank. After vortexing, 200 µl of the diluted antiserum was added and mixed briefly again. To allow a complete reaction of the antibodies with the benzodiazepine and/or tracer, the tubes were stored for 60 min at room temperature. After that 400 ml saturated ammonium sulfate was added in order to stop the reaction and to separate the free tracer fraction from the antibody-captured tracer fraction. The tubes were centrifuged at 4000 rpm for 20 min. The supernatant was discarded and the pellet was washed with 900 µl of 50% saturated ammonium sulfate after brief mixing on a vortex. The tubes were again centrifuged at 4000 rpm for 20 min, the supernatant was discarded and the pellet redissolved in 300 µl water. To measure the activity of the ^{3}H-flunitrazepam, 3 ml scintillation-solution (Quickszint-402, Zinsser-Analytic) was added and finally the tubes were vortexed and placed in a liquid scintillation counter (LKB-Wallac, 12111 rackbeta liquid scintillation counter). The raw data was calculated in $\%B/B_0$ and Fig. 2 displays the curves for flunitrazepam, 3-hydroxyflunitrazepam and nitrazepam. The cross-reactivity of various benzodiazepines was measured and the results are listed in Table 1.

Another technique to separate the free tracer fraction from the antibody-captured tracer fraction was the use of sheep-anti-rabbit-IgG Dynabeads (polystyrene-magnetic beads). The reactive part of the antibodies in the antiserum which bound the ^{3}H-flunitrazepam, was immobilised onto a magnetisable solid phase via a sheep antibody against rabbit IgG and was separated in a magnetic field (Dynal MPC-magnetic particle concentrator). The comparison of the 2 techniques for separation of bound tracer and free tracer is displayed in Fig. 3. The two techniques led to nearly the same results, so for further determinations the very cheap ammonium sulfate precipitation was used.

Flunitrazepam determination in whole blood

The aim of the study was the specific determination of therapeutic concentrations of flunitrazepam in whole blood in a simple way. The easiest way is the direct use of whole blood but this procedure is not possible when using a tritium-labelled tracer because haemoglobin absorbs the radiation. An easy extraction as a batch process was therefore made: 1 ml whole blood (spiked standards) was diluted with 1 ml 1 M TRIS buffer solution (pH 10) and extracted with 1 ml polystyrene suspension (Amberlite XAD, 10% in TRIS buffer solution). The samples were mixed for 10 min in an overhead shaker and centrifuged. The supernatant was discarded, and the residue washed twice with 5 ml TRIS buffer solution. Desorption of bound benzodiazepines was achieved by extracting the polystyrene residue twice with ethyl acetate. The ethyl acetate phases were separated in a clean tube, evaporated to dryness and the residue redissolved in 1 ml PBS.

Calibration standards made by spiking blank whole blood, and samples were extracted in the same way. Figure 4 shows a typical calibration curve of spiked whole blood samples using this extraction procedure. The curve displayed linearity between the therapeutic relevant range of 1–50 ng/ml whole blood. The extraction was controlled by GC-ECD determination of the benzodiazepines and could be set to 81–92%. It was not the aim to tune the extraction to near 100%, because the standards and samples were extracted in the same way and the determination was sensitive enough. The detection limit, calculated as blank – 3σ, could be set at < 0.1 ng/g whole blood.

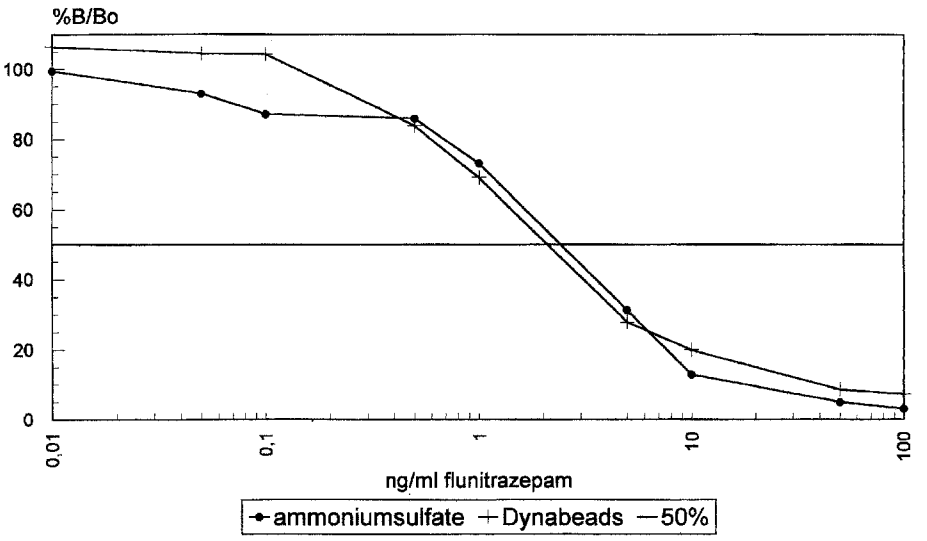

Fig. 3 Comparison of the separation of bound tracer by ammonium sulfate precipitation and polystyrene-magnetic beads (Dynabeads)

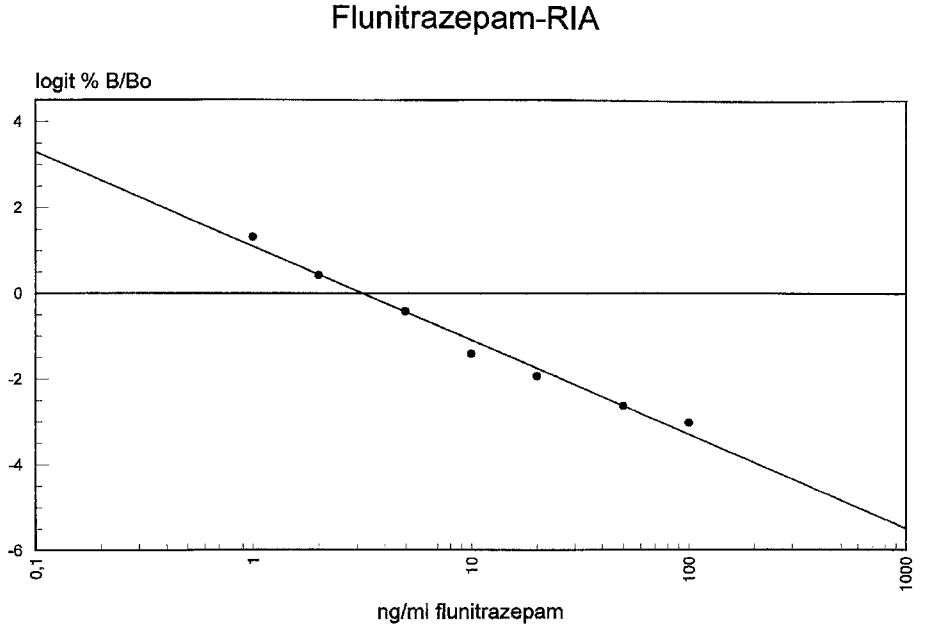

Fig. 4 Logit-log calibration curve of spiked whole blood samples

Results and discussion

For the detection of flunitrazepam in whole blood, flunitrazepam was derivatized in position 3 of the benzodiazepine ring, in order to protect the characteristic substituents. By coupling to carrier proteins like BSA and KLH, an immunogen was synthesized. Rabbits were immunized with the immunogens, and blood was collected at several time points. No further increase in the antibody titre was found after $7^1/_2$ weeks of further immunisation. The antisera were tested in a heterogeneous, competitive immunoassay with ^{3}H-flunitrazepam as a tracer. The cross-reactivity of various benzodiazepines was tested and was < 1%, with the exception of 3-hydroxyflunitrazepam (18%) and demethylflunitrazepam (3%). The high cross-reactivity of 3-hydroxyflunitrazepam could be explained by its similarity to the structure of the derivatized flunitrazepam-hapten. The comparison of the cross-reactivities showed that all substituents of the benzodiazepine ring were the most important parts of the epitopes, responsible for the specificity of the antibodies. The N_1-methyl group, the 7-nitro-substituent and the 2′-fluor substituent were suggested as responsible antigen determinants, because the reduction of the strongly antigenic nitro-group to the amino-function, as in aminoflunitrazepam, leads to an absolute decrease in the cross-reactivity.

For the determination in whole blood, a simple extraction procedure with a polystyrene suspension in TRIS-buffer was made. Spiked whole blood samples were used for calibration of the assay, so that samples and calibration standards were handled in the same way. The procedure allows the determination of flunitrazepam in whole blood

in the therapeutic range. The statistical detection limit (blank – 3σ) was < 0.1 ng/g whole blood. The main metabolites demethylflunitrazepam and aminoflunitrazepam were not detected by this method, but it was not possible to obtain antibodies which recognize a benzodiazepine with its metabolites and without cross-reactivity to other benzodiazepines.

The method presented is an easy way for the determination of therapeutic concentrations of flunitrazepam in whole blood. It shows a way for the development of an immunochemical test system specific for one drug problem, a single test for flunitrazepam. This solution of the problem to determine therapeutic flunitrazepam concentrations in whole blood is no universal solution for other drugs, but it could terminate the discussion of false-negative results caused by determination of flunitrazepam in body fluids with commercially available immunoassays.

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