Screening of benzodiazepines with special reference to lorazepam

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1. Introduction

Benzodiazepines are widely prescribed drugs for the treatment of anxiety, epilepsy and insomnia. They are liable to abuse and capable of producing impairment of driving. Benzodiazepines are frequently encountered in drugs and driving cases.

The most common screening techniques (immunological techniques, GC/MS and GC/NP-detector) are poor in detecting "low level benzodiazepines" like lorazepam in biological fluids. These analytical difficulties may give false negative results in screening tests and erroneously too low incidence of lorazepam in epidemiological studies.

We present here a screening procedure for benzodiazepines which has been built up with special reference to low dose benzodiazepines such as lorazepam. In this procedure TLC acts as the main screening method. Final identification is performed by gas chromatograph combined with EC and NP detectors.

2. Methods

2.1 Screening of benzodiazepines in urine by TLC

Glucuronide conjugates of benzodiazepines are released in urine by acid hydrolysis (conc. HCl at 100 °C) to yield benzophenones. They are extracted with petroleum ether. After evaporation of the organic phase, the residue is dissolved in 50 ul of ethanol and applied on the DC-Alufolien TLC-plates coated with Kieselgel 60.

Toluene is used as mobile phase. The plates are dried prior to visualization and then sprayed with following reagents:
1. 9 M Sulfuric acid
2. 1 % Sodium nitrite in water
3. 5 % Ammonium amidosulphonate
4. 1 % N-(1-naphtyl)-ethylenediamine dihydrochloride in water-acetone (8:2).

If the drugs are found in urine, respective blood samples will be analyzed. Because several benzodiazepines can produce the same benzophenone, final identification has to be confirmed by another technique.

2.2 Identification of benzodiazepines in blood by GC

Samples of 1ml of blood, control solutions and working standards are shaken with 1 ml of 0.5M NaH₂PO₄ and 5 ml of n-hexane:dichloromethane (70:30) containing flurazepam as internal standard. Flurazepam was chosen as internal standard because it is not marketed in our country. After centrifugation, the aqueous layer is discarded and the organic layer evaporated.

The gas chromatographic analyses are carried out with a capillary gas chromatograph (Hewlett Packard 5890 Series II) combined both with EC- and NP-detectors. Fused silica capillary columns, coated with HP-5 (5 % phenylmethylsilicone, film thickness 0.17 um, 25 m x 0.31 mm I.D.) are connected to the same injector. Injection is done in splitless mode. The oven temperature is held initially at 200 °C for 1 min and increased thereafter up to 290 °C at 10 °C/min.

3. Results

3.1. TLC

Hydrolysis product of lorazepam is aminodichlorobenzophenone (ACCB), which gives a violet spot at hRf 56. Methylaminochlorobenzophenone (MACB), the hydrolysis product of diazepam, medazepam and temazepam can be seen as a yellow spot at hRf 76 before spraying. Aminochlorobenzophenone (ACB) is the hydrolysis product of nordiazepam, oxazepam and chlordiazepoxide and produces a violet spot at hRf 47. The aminonitrobenzophenone (ANB) (red at hRf 16), aminochlorofluorobenzophenone (ACFB) (violet at hRf 50) and aminonitrochlorobenzophenone (ANCb) (red at hRf 25) are the hydrolysis products of nitrazepam, midazolam and clonazepam, respectively.

The detection limits for benzodiazepines as benzophenones are the follows: lorazepam 0.1 µg; clonazepam and nitrazepam 0.01 µg; chlordiazepoxide, diazepam, nordiazepam, oxazepam and temazepam 1 µg. Triazolam and alprazolam do not produce benzophenone at all.
EC- and NP-chromatograms of an extracted blood standard containing a mixture of benzodiazepines are shown in figure 1. The EC-detector responds to lorazepam, clonazepam, triazolam and alprazolam better than the NP-one. Especially for lorazepam, the EC detector is essential. Oxazepam, diazepam and nordiazepam produce easily detectable peaks in both channels, although the EC-detector is more sensitive.

The NP detector in turn is necessary to detect chlordiazepoxide and medazepam. These compounds produce no peaks at all in the EC-channel at moderate blood levels. Thermolabile temazepam produces two separate peaks.

The detection limits in blood range from 10 ng/ml (oxazepam, lorazepam, diazepam, nordiazepam, chlordiazepoxide, temazepam and triazolam) to 50 ng/ml (oxazepam).

Figure 1. EC (left) and NP (right) chromatograms of an extracted plasma standard containing a mixture of benzodiazepines.

Peaks: 1 = oxazepam (1 mg/l); 2 = lorazepam (0.1 mg/l); 3 = diazepam (1 mg/l); 4 = nordiazepam (1 mg/l); 5 = chlordiazepoxide (1 mg/l); 6 = temazepam (1 mg/l)(first peak); 7 = temazepam (second peak); 8 = flurazepam (as internal standard); 9 = nitrazepam (0.1 mg/l); 10 = clonazepam (0.1 mg/l); 11 = triazolam (0.1 mg/l).

Columns: SE-54, 25 m x 0.31 mm I.D., fused silica. Injection: splitless at 280 °C. Detector temperatures 300 °C.

Oven temperature: initially 200 °C for 1 min., rate 10 °C, final temperature 290 °C.
4. Discussion

The TLC technique, used for decennies, is very selective and sensitive for benzophenones, hydrolysis products of benzodiazepines. The low level benzodiazepines; lorazepam, clonazepam, nitrazepam and midazolam in the urine specimens e.g. of suspected DUI-drivers can be detected using this technique. Alprazolam and triazolam, however, do not produce benzophenones by acid hydrolysis and so GC/EC is used for their identification.

Although TLC in drug screening has been replaced in many laboratories by GC/NP and HPLC, the recent instrumental development in TLC technique offers new possibilities. Nowadays TLC can be automatized e.g. with automated sample application, instrumental modes of development, and computerized identification with retardation factor, color reactions and spectra.

The dual column GC-analysis has been used in order to increase the number of drugs detected in a single run. Specific and sensitive EC- and NP-detectors combined in dual-column system are a powerful tool for detecting and determining benzodiazepines. EC-detector is essential for determination of lorazepam and other low dose benzodiazepines.