Difficulties and Pitfalls in the Toxicological Analysis of Benzodiazepines

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INTRODUCTION

Recently, benzodiazepines have become one of the most important groups of so-called toxicologically significant substances. Not only because of their frequent occurrence as positive findings in forensic as well as in clinical toxicogical analyses but also due to the high incidence of abuse. More than 3800 samples (cases) were analysed for benzodiazepines over a period of the last five years (2001- 2005) in our department. Around one third were positive. Thus, benzodiazepines are currently the most frequently found substance in our toxicological routine.
Although identification and determination of benzodiazepines in biological material are analytically well managed, certain problems sometimes do arise, especially in the interpretation of the results. Such problems in some laboratories can lead to incorrect conclusions.

METHODS

Screening analysis of benzodiazepines (BZD.

Immunoassays have become the most commonly used as routine preliminary tests for benzodiazepines in urine in most toxicological laboratories in the Czech Republic. Their major advantage is that they do not require any sample preparation. In our laboratory we use Fluorescence Polarisation Immunoassay (FPIA). The originally employed ADx system has been replaced by the AxSYM system (Abbott Laboratories) with a cut-off value set to 200ng/ml.

Thin layer chromatography (TLC)

TLC is suitable for the detection of parent forms of BZD, especially in gastric contents, as well as for screening of some BZD metabolites, particularly in urine. Using HPTLC and the Bratton-Marshall reagent enables sensitive detection of the metabolites of flunitrazepam, clonazepam and nitrazepam as well as of some other metabolites (in the form of benzophenones) after acid hydrolysis.

Gas chromatography with electron capture detector (GC-ECD

GC-ECD is used for simple, rapid, sufficiently sensitive and cost effective determination of BZD in blood (serum).
Sample preparation: so called “freeze-out method”: 0,5ml serum + internal standard (lorazepam) + 0,05 ml 2M TRIS buffer pH 9 + 0,15 ml butyl acetate mixed, centrifuged and frozen-out (1 hour, - 20°C)

Instrumentation: Gas chromatograph AutoSystem XL (Perkin Elmer) with ECD, capillary column DB5 (15m x 0,25mm x 0,25µm), temperature programme: 180°C, 20°C/min., 250°C, then 10°C/min., 280°C (10 min.)

Gas chromatography-mass spectrometry (GC-MS)

GC-MS is suitable for the selective detection of BZD metabolites in urine after enzymatic hydrolysis using beta-glucuronidase, solid phase extraction (using SPE - Bond Elut Certify, Varian) and derivatization (silylation).

Instrumentation: Gas chromatograph Trace GC, Mass spectrometer Polaris Q (Thermo Finnigan), capillary column RTX-5MS (15m, 0.25mm x 0.25µm), temperature programme: 210°C (1 min.), 20°C /min., 290°C (10 min.), full scan mode (40 - 450 AMU).

RESULTS AND DISCUSSION

Table 1 and the graphs 1) and 2) show the total number of analyses on BZD carried out in our department over the five-year period, including the positive findings. The number of false negative results of immunochemical screening with subsequent positive findings is another monitored parameter together with the number of quantitative analyses.

Table 1: The number of analyses on BZD carried out in our department over the five-year period (2001- 2005).

	Total number of analyses focused on BZD			Number of positive results			Number of quantitative analyses			Number of false negative results		
Year	Persons Dead	Alive	Together	Dead	Alive	Together	Dead	Alive	Together	dead	live	Together
2001	160	461	**621**	18	171	**189**	10	53	**63**	1	15	**16**
2002	187	547	**734**	26	197	**223**	20	84	**104**	4	15	**19**
2003	186	609	**795**	27	233	**260**	20	61	**81**	4	11	**15**
2004	233	573	**806**	41	182	**223**	35	64	**99**	8	13	**21**
2005	257	590	**847**	36	194	**230**	29	74	**103**	9	18	**27**
Together	1023	2780	3803	148	977	1125	114	336	450	26	72	98

Despite the undeniable advantages of immunoassays for BZD screening of urine there are drawbacks. These can be of major importance, especially when interpreting the results. The main problems are both false negative and false positive results.

False positivity was sporadically observed in cases when a high concentration of indomethacin, cocaine, methadone, sertraline or trazodone was determined in biological material. For this reason positive results obtained from immunochemical methods should always be confirmed by another independent method. There are, however, some laboratories in our country (mainly biochemical) which do not respect this general rule.

Figure 1: The number of requested analyses for benzodiazepines and number of positive findings

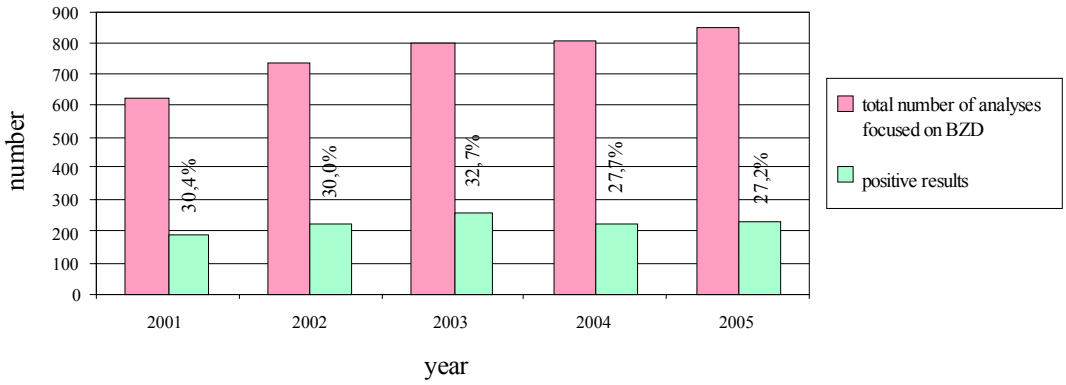

Figure 2: Number of positive results, quantitative analyses and number of false negative results

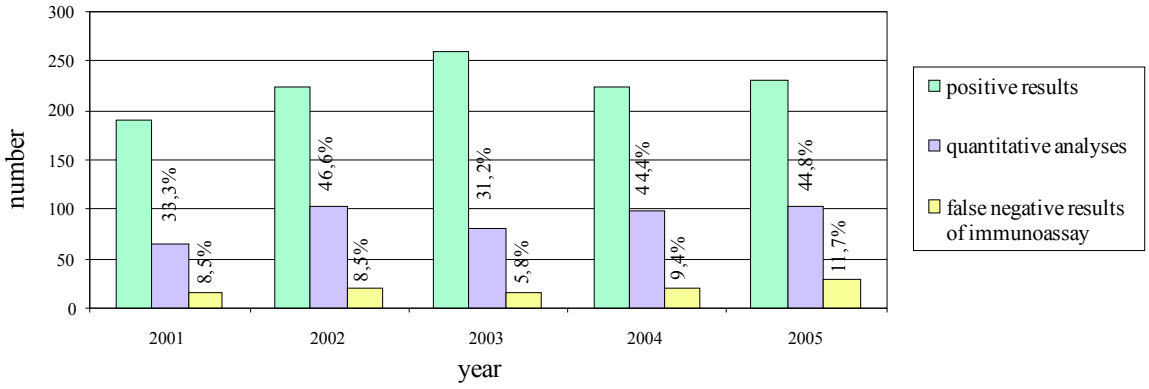

Table 2: shows some interesting examples of false negativity (FPIA results were lower than the cut-off value).

Table 2: Some interesting examples of false negative results for benzodiazepines

group	ADx or AxSYM (U)	TLC (U) or GC-MS (U)	TLC (GaC)	GC-ECD (B)	Concentration (mg/L)
A	24	negative	negative	diazepam	0,02 -therap.conc.
	152	negative	negative	flunitrazepam	0,01-therap.conc.
	43	negative	negative	bromazepam diazepam nordiazepam	0,13-therap.conc 0,12-therap.conc. 0,17-therap.conc.
	75,51	negative	negative	bromazepam	0,10-therap.conc.
B	17	7-aminoflunitrazepam	negative	flunitrazepam	0,13-toxic conc.
	85,18	negative	negative	clonazepam	0,42-toxic.conc
	33,07	negative	midazolam	midazolam clonazepam	0,60 terap.-toxic. 0,11 toxic.conc.
	188,27	7-aminoclonazepam α-hydroxyalprazolam	negative	clonazepam alprazolam	0,14 toxic.conc. 0,05 terap.conc.

U – Urine, GaC - Gastric contens, B - Blood

False negativity obtained from immunochemical testing occurred when the urine contained metabolites not only of bromazepam, flunitrazepam, nitrazepam and clonazepam, but also of diazepam and alprazolam. This is a significant problem because the false negativity occurs even in cases of intoxication through some of the above mentioned BZD.

Table 2 shows some interesting examples of false negativity (FPIA results were lower than the cut-off value).

The first part (A) includes cases where not only FPIA analysis of urine but also TLC analysis of urine and gastric contents proved negative. Positive results were not found until examination of a blood sample using the GC-ECD method.

The second part (B) shows examples of toxic and therapeutical-toxic concentrations of BZD which were determined following a negative prior FPIA analysis of urine and, in some cases, also a negativeTLC analysis of urine and gastric contents.

CONCLUSIONS

The results and experiences presented here show that despite the well-known advantages of immunoassays in screening analysis of urine for drugs of abuse (including BZD), it is necessary to confirm practically all benzodiazepine results obtained in this way using subsequent methods. Immunochemical screening of urine should be always followed by identification of BZD and their metabolites in urine or better in both urine and gastric contents using e.g. TLC, GC-MS and subsequent BZD quantitation in blood, for example using GC-ECD.

Only complex toxicological analysis of biological material (urine, gastric contents and blood) using immunochemical as well as chromatographical methods permits toxicological laboratories to give valid interpretation of results, thus contributing to objective evaluation of the level of intoxication in forensic as well as clinical practice.