ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
18 (1998) 681–687

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Comparison of different immunoassays and GC-MS screening of benzodiazepines in urine

M. Augsburger *, L. Rivier, P. Mangin

Institut Universitaire de Médecine Légale, rue du Bugnon 21, 1005 Lausanne, Switzerland

Received 15 May 1998; received in revised form 1 September 1998; accepted 12 September 1998

Abstract

A total of 53 urine samples were tested by different immunoassay methods and by gas chromatography/mass spectrometry to determine repeatability of the different methods and to assess whether the immunoassays performed on samples obtained from elderly patients of the emergency section could be considered as reliable enough for identifying a benzodiazepine consumption. Repeatability was excellent for GC/MS and good for immunoassays. The specificity was not different for the three immunoassays (96%). The sensitivity varied from 36, 64 to 75% for OnLine, RIA Immunalysis and RIA DPC, respectively. An other difference between immunoassays and GC/MS was the ability of GC/MS to detect lorazepam and low concentrations of benzodiazepines whereas immunoassays did not.

Keywords: Benzodiazepines; Screening; Radioimmunoassays; KIMS; GC-MS

1. Introduction

Benzodiazepines are therapeutically used for hypnotic, anxiolytic, anticonvulsant and muscle relaxant effects. These drugs are among the most frequently prescribed medications encountered in our usual practice. A high incidence of benzodiazepines has been reported in a retrospective study of suspected impaired drivers [1].

The detection of benzodiazepine consumption must combine specificity, sensitivity and repeatability. Urine concentration of benzodiazepines and benzodiazepine metabolites can differ by several orders of magnitude, depending in particular of the drug, the daily dosage, the metabolism and the time of sample collection. The great number of benzodiazepines available and their metabolites makes the development of a comprehensive screening method difficult. Methods that have been proposed for detection and quantification of benzodiazepines in biological fluids include radioimmunoassays [2], radioreceptor assays [3], fluorescence polarization immunoassays (FPIA) [4], kinetic interaction of microparticles in a solution (KIMS), enzyme multiple immuno technique (EMIT) [5,6], high-performance liquid chromatography (HPLC) with UV detection [7], HPLC with photodiode array detection (DAD) [8], gas chromatography (GC)

* Corresponding author. Tel.: +41-21-3147070; fax: +41-21-3147090; e-mail: marc.augsburger@inst.hospvd.ch.

0731-7085/98/$ - see front matter
PII: S0731-7085(98)00266-0

with electron-capture and/or nitrogen-phophorus detection [9,10], GC/mass spectrometry (MS) [11–13] and negative chemical ionization (NCI) GC/MS [14].

Because urine collection is less invasive, benzodiazepine screening procedures are often designed for use with this matrix. Only few of the parent benzodiazepines appear in urine; therefore, the detection of the commonly excreted metabolites is crucial for a reliable identification of benzodiazepine consumption.

Different immunoassays have been developed for identifying benzodiazepine consumption. These tests are often presented as very attractive methods for the detection of benzodiazepine consumption because of their simplicity, low cost, specificity and rapid turnaround time. In clinical practice, immunoassay results may be sufficient to verify a diagnostis and to continue or begin a treatment, however, for forensic cases, another independant analytical method such as GC/MS must be used in order to confirm a positive test.

Previous studies have investigated the reaction of some benzodiazepines and some of their metabolites with different immunoassays and evaluated different commercial urine benzodiazepine immunoassays [4–6,14,15]. Here we evaluated two commercial urine benzodiazepine immunoassays and a GC/MS screening in double-blind study with urine samples obtained from elderly patients of the emergency section. Moreover we compared results provided by three commercial urine benzodiazepine immunoassays with results obtained with GC/MS. This study allowed us to evaluate the repeatability, the sensitivity, the specificity and the efficiency of the diagnostis of various urine benzodiazepine immunoassays.

2. Materials and methods

2.1. Biological samples

A total of 53 urine samples were obtained from patients of the emergency section of the CHUV hospital, Lausanne, Switzerland. Patients were included in a study of hypnotic consumption and the maintenance of independance in the elderly (conducted by Dr B. Yersin, emergency section CHUV Lausanne). All specimens were aliquoted in two groups: sample (group 1) and duplicate (group 2) and stored at −20°C. Samples were numbered from 1001 to 1053 and duplicates were randomly numbered from 1054 to 1106. The correspondence between samples and duplicates was indicated to analysts only after data had been collected.

2.2. Immunoassays

Three immunoassay methods, (a) double-antibody radioimmunoassay (RIA) Diagnostic Products (DPC, Los Angeles, CA), (b) RIA Immunalysis (Immunalysis, San Dimas, CA) and (c) Roche Abuscreen OnLine system (KIMS) on COBAS MIRA (Roche Diagnostic Systems, Nutley, NJ) were tested. The kits were used according to manufacturer's specifications. A summary of the cutoff calibrator and other immunoassay kit characteristics is shown in Table 1. Quality-control specimens provided by the manufacturers were analyzed each day assays were performed. Acceptability of control values was determined according to the manufacturers' instructions.

2.3. GC/MS

After adding internal standard (phenazine), urine samples (2 ml) were hydrolysed with 1 ml HCl (32%) for 30 min at 100°C, then basified with

Table 1
Characterization of benzodiazepine immunoassays examined (RIA DPC, RIA Immunalysis and Abuscreen OnLine)

	RIA DPC	RIA Immunalysis	OnLine
Cutoff (ng ml^{-1})	100	50	100
Standard	oxazepam	oxazepam	nordiazepam
Volume required (μl)	50	25	12
Isotope/wavelength	^{125}I	^{125}I	500 nm

Table 2
Significant ions (m/z) in mass spectra, retention index (RI) and detection limit (LOD) of benzophenones and acetylated benzophenones (ac)

Benzophenone	Ion (m/z)	RI(IS)[a]	RI(Pfleger)[b]	LOD (ng ml^{-1})	Benzodiazepine consumption
ACB ac	230, 273	1.444	2245	25	Oxazepam, nordiazepam, clorazepate, …
MACB	245, 228	1.329	2100	50	Diazepam, temazepam, ketazolam, …
CMCB	285, 270	1.565	2410	50	Prazepam
ANCB	241, 276	1.685	2470	250	Clonazepam
ABBP ac	249, 318	1.612	2490	150	Bromazepam
MNFB	274, 257	1.594	2370	5	Flunitrazepam
MAAFB ac	205, 328	1.927	2870	150	Flunitrazepam
DAFB ac	314, 230	1.858	2715	150	Flunitrazepam
ANFB	260, 213	1.560	2335	150	Flunitrazepam
DACFB	86, 348	1.692	2555	50	Flurazepam
ACFB ac	248, 291	1.408	2195	2.5	Flurazepam
HEACFB ac	262, 335	1.639	2470	5	Flurazepam
ADB ac	230, 307	1.526	2300	25	Lorazepam, lormetazepam, …
MADCB	244, 279	1.435	2220	25	Lormetazepam, …
ANB	241, 195	1.600	2365	150	Nitrazepam
DAB ac	296, 212	2.004	2985	150	Nitrazepam
MTDCB	296, 331	1.923	2865	150	Triazolam

[a] RI(IS): retention time of benzophenone/retention time of internal standard (IS, phenazine).
[b] RI(Pfleger): RI according to [16].

1.25 ml NaOH (10M) and 3 ml ammonia buffer (pH 9.5). The drugs were extracted into a 3 ml mixture of chloroform and isopropyl alcohol (9:1, v/v), evaporated to dryness and acetylated 30 min at 60°C with a 100 μl mixture of acetic acid anhydride and pyridine (3:2, v/v). After evaporation of the acetylation mixture the residue was dissolved in 0.1 ml ethyl acetate and 1 μl was injected into the GC/MS. GC/MS analysis was performed using a Hewlett-Packard (HP) 6890 gas chromatograph fitted with a HP-5MS capillary column (30 m, 0.25 mm I.D., 0.25 μm film thickness) and coupled with HP 5973 mass selective detector.

The column temperature was programmed from an initial 100°C, after 1 min hold, to 200°C at 20°C min^{-1}, then to 300°C at 15°C min^{-1}. MS source temperature was 230°C and MS quadrupole temperature was 150°C. Spectra were collected over a mass range from 50 to 600 amu using 70 eV electron energy.

A macro was developed for the screening of benzophenones produced by hydrolysis of benzodiazepines. Two specific ions were shown for each benzophenone (Table 2) around the retention time of the substance. The limit of detection (LOD) of benzophenones, defined by a signal-to-noise ratio of 3 for both significant ions, was presented in Table 2. The presence of any of these compounds can be confirmed by comparison of the peak full mass spectra with reference spectra. Chromatograms in Fig. 1 corresponded to a urine specimen obtained from hospitalized patient. Results suggested the presence of the acetylated oxazepam benzophenone, which was confirmed by comparison of the peak full mass spectra with reference spectra (Fig. 2).

The GC/MS screening targeted consumption of oxazepam (2-amino-5-chlorobenzophenone (ACB)), nordiazepam (ACB), clorazepate (ACB), diazepam (ACB and 2-methylamino-5-chlorobenzophenone (MACB)), chlordiazepoxide (ACB), temazepam (ACB and MACB), ketazolam (ACB and MACB), camazepam (ACB and MACB), prazepam (ACB and 2-cyclopropyl-methylamino-5-chlorobenzophenone (CMCB)), flurazepam (2-diethylamino-ethylamino-5-chloro-2'-fluorobenzophenone (DACFB), 2-amino-5-chloro-2′-fluorobenzophenone (ACFB) and 2-hydroxyethylamino-5-chloro-2′-flurorbenzophenone (HEACFB)),

flunitrazepam (2-methylamino-5-nitro-2′-fluorobenzophenone (MNFB), 2-methylamino-5-amino-2′-fluorobenzophenone (MAAFB), 2,5-diamino-2′-fluorobenzophenone (DAFB) and 2-amino-5-nitro-2′-fluorobenzophenone (ANFB)), lorazepam (2-amino-5,2′-dichlorobenzophenone (ADB)), lormetazepam (ADB and 2-methylamino-2′,5-dicholobenzophenone (MADCB)), delorazepam (ADB), oxazolam (ACB), bromazepam (2-(2-amino-5-bromo-benzoyl)-pyridine (ABBP)), nitrazepam (2-amino-5-nitrobenzophenone (ANB) and 2,5-diaminobenzophenone (DAB)), medazepam (ACB and MACB), clonazepam (2-amino-5-nitro-2′-chlorobenzophenone (ANCB)), triazolam (2-methyltriazolo-2′,5-dichlorobenzophenone (MTDCB)).

3. Results and discussion

Of the 53 urine samples, 28 contained benzodiazepines as determined by GC/MS. Among these 28 urine samples, one benzophenone was detected in 20 cases, two benzophenones were detected in seven cases and three benzophenones were detected in one case. In positive samples, the following benzophenones were detected by GC/MS: ACB ($n = 19$), MACB ($n = 2$), CMCB ($n = 1$),

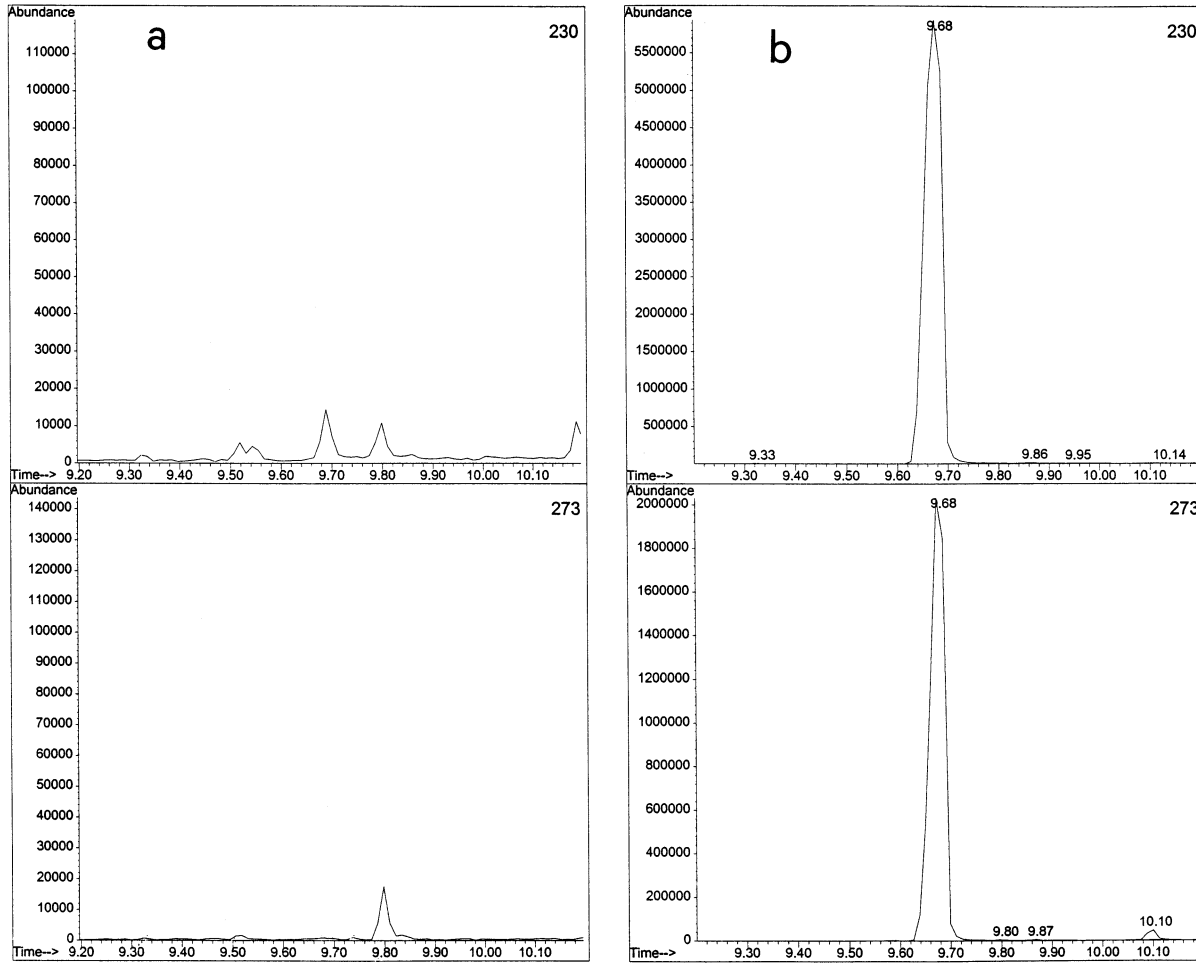

Fig. 1. Selected ion chromatograms of blank urine sample (a) and urine sample obtained from hospitalized patient (b). Ion m/z 230 and ion m/z 273 are specific for acetylated oxazepam benzophenone (ACB).

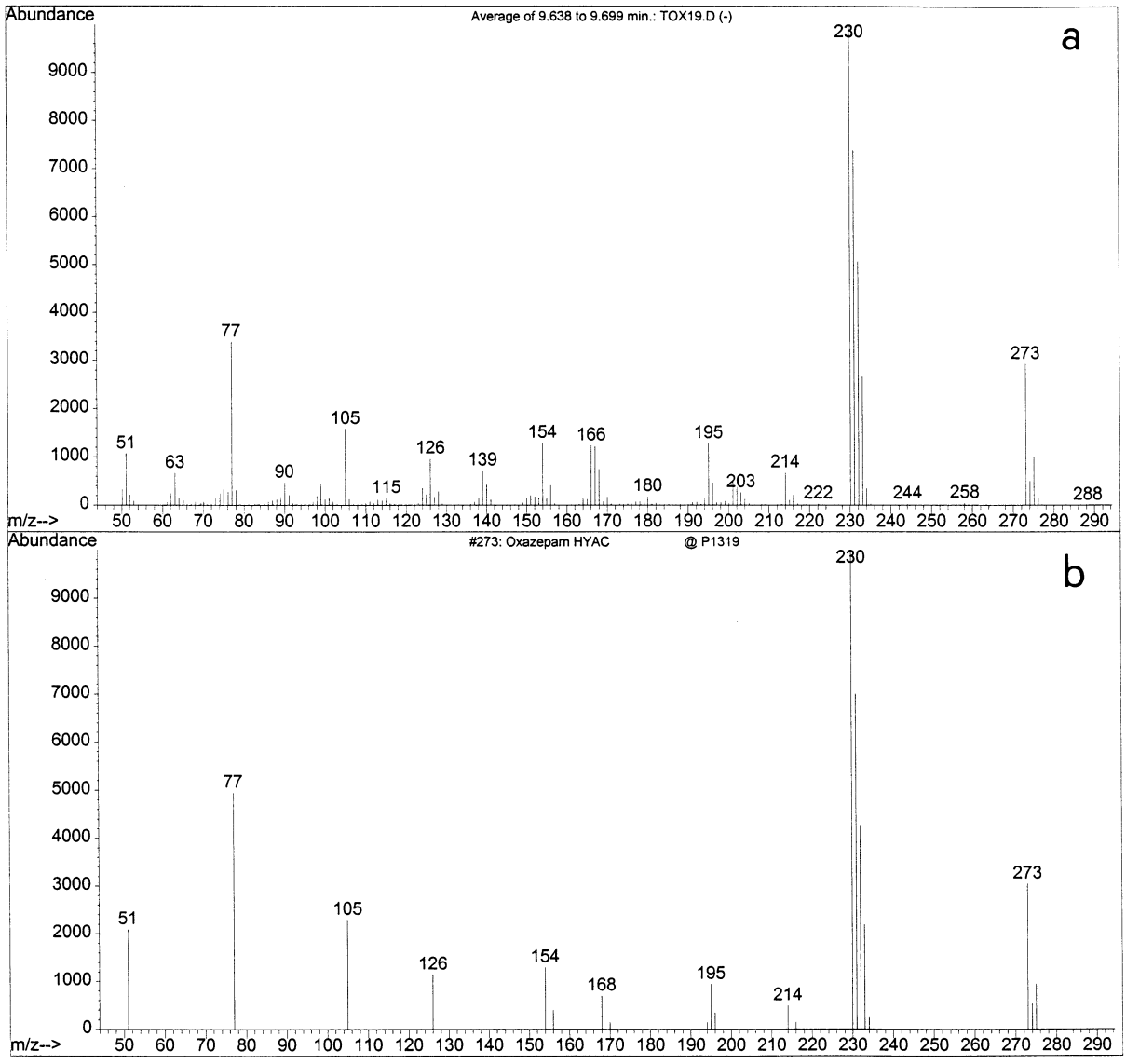

Fig. 2. Comparison of the peak full mass spectra (a) from Fig. 1 with reference spectra (b) according to [16].

HEACFB ($n = 2$), ACFB ($n = 4$), ADB ($n = 4$), MADCB ($n = 1$) and ABBP ($n = 4$).

Sample group and duplicate group were compared for RIA DPC, COBAS OnLine and GC/MS analyses (Table 3). For GC/MS analyses, all sample group results were exactly identical with duplicate group results (negative/positive and identification of the benzophenone). One and two cases did not match between sample group and duplicate group for RIA DPC and OnLine analyses, respectively. Results of these cases were just above the cutoff limit for one group and just below for the other group.

The number of true positives (TP), false negatives (FN), false positives (FP) and true negatives (TN) was determined by comparison the immunoassay results (RIA DPC, RIA Immunalysis and COBAS OnLine) with the results provided by

Table 3
Contingency tables for benzodiazepine double-blind study. Samples vs. duplicates for RIA DPC, COBAS OnLine and GC/MS analyses

		Duplicates					
		RIA DPC		COBAS On-Line		GC/MS	
		+	−	+	−	+	−
Samples	+	21	1	10	1	28	0
	−	0	31	1	41	0	25

GC/MS (Table 4). Sensitivity and specificity were calculated according to the following formulas: sensitivity = TP/(TP + FN) and specificity = TN/(TN + FP). The specificity was not different for the three immunoassays (96%). The sensitivity varied from 36, 64 to 75% for COBAS OnLine, RIA Immunalysis and RIA DPC, respectively.

These results illustrate an important problem when urine benzodiazepine screenings are used for the monitoring of prescription compliance. A negative result given by an immunoassay does not necessarily indicate that the patient is noncompliant. Moreover for forensic cases these results demonstrate that benzodiazepine immunoassay screenings have to be completed with GC/MS screenings.

All cases with GC/MS positive results for lorazepam and lormetazepam were negative with immunoassay methods. This may be explained by the poor cross-reactivity of lorazepam and lormetazepam in the immunoassay systems. Other false negative cases were benzodiazepine low concentration samples. Previous publications reported that enzymatic hydrolysis was necessary before immunoassay screening tests for oxazepam and other benzodiazepines [15]. Because we used acid hydrolysis only before GC/MS analysis and no glucuronidase before immunoassay, we cannot rule out that hydrolysis might increase the sensitivity of the immunoassays for benzodiazepines other than those related to oxazepam (e.g. flurazepam or lorazepam). Nevertheless other publications reported lack of cross-reactivity of certain immunoassays to lorazepam independently of hydrolysis [14], that could be compared with present results.

4. Conclusion

Both RIA and OnLine techniques, when used as intended by the manufacturers with real urine samples, present good repeatability and specificity but poor to bad sensitivity, in particular for the detection of lorazepam, lormetazepam and low concentrations of benzodiazepines. For this reason we conclude that (a) as previous report [14,15], immunoassays are unreliable for the detection of intake of therapeutic doses of benzodiazepines, and (b) GC/MS stays the most reliable method for the screening of benzodiazepines in urine.

Acknowledgements

The authors thank Carole Mayor for her skilled technical assistance and Dr Bertrand Yersin and Dr Thierry Buclin for providing and collecting urine samples.

Table 4
Contingency tables for benzodiazepine immunoassays

		Duplicates					
		RIA DPC		COBAS OnLine		RIA immunanalysis	
		+	−	+	−	+	−
GC/MS	+	21	7	10	18	18	10
	−	1	24	1	24	1	24

References

[1] M. Augsburger, L. Rivier, Forensic Sci. Int. 85 (1997) 510–523.
[2] R. Dixon, Immunochem. Technol. 84 (1982) 490–575.
[3] B. Borggaard, I. Joegensen, J. Anal. Toxicol. 18 (1994) 243–246.
[4] J. Becker, A. Correl, W. Koepf, C. Rittner, J. Anal. Toxicol. 17 (1993) 103–108.
[5] A.D. Fraser, R. Meatherall, J. Anal. Toxicol. 20 (1994) 217–223.
[6] O. Beck, Z. Lin, K. Brodin, S. Borg, P. Hjemdahl, J. Anal. Toxicol. 21 (1997) 554–557.
[7] R. Gill, B. Law, J.P. Gibbs, J. Chromatogr. 356 (1986) 37–46.
[8] W.E. Lambert, E. Meyer, Y. Xue-Ping, A.P. De Leenheer, J. Anal. Toxicol. 19 (1995) 35–40.
[9] P. Lillsunde, T. Seppälä, J. Chromatogr. Biomed. Appl. 533 (1990) 97–110.
[10] H. Gjerde, E. Dahlin, A.S. Christophersen, J. Pharm. Biomed. Anal. 10 (5) (1992) 317–322.
[11] W.A. Joern, J. Anal. Toxicol. 16 (1992) 363–367.
[12] C. Moore, G. Long, M. Marr, J. Chromatogr. B 655 (1994) 132–137.
[13] R. Meatherall, J. Anal. Toxicol. 18 (1994) 369–381.
[14] R.L. Fitzgerald, D.A. Rexin, D.A. Herold, Clin. Chem. 40 (3) (1994) 373–380.
[15] O. Beck, P. Lafolie, P. Hjemdahl, S. Borg, G. Odelius, P. Wirbing, Clin. Chem. 38 (2) (1992) 271–275.
[16] K. Pfleger, H.H. Maurer, A. Weber, Mass Spectral and GC Data of Drugs, Poisons, Pesticides and Their Metabolites, Part 1 (Methods, Tables, Indexes), Second, revised and enlarged edition, VCH Verlagsgesellschaft mbH, Weinheim, 1992.