BENZODIAZEPINE FINDINGS IN BLOOD AND URINE BY GAS CHROMATOGRAPHY AND IMMUNOASSAY

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ABSTRACT: Gas chromatography (GC) and immunoassay techniques applied to blood and urine specimens were compared for the screening of benzodiazepines in post-mortem forensic toxicology. Five hundred and six such successive medical examiner's cases were selected, where both urine and blood samples were available. Urine specimens were analysed by Emit® d.a.u.™ Benzodiazepine Assay. Blood and urine samples were screened for benzodiazepine drugs and their metabolites by an automated dual-column GC method. The results suggest that the present GC method for the blood seems to be a good alternative to the common combination of urine immunoassay followed by quantitative analysis of blood by chromatography.

KEY WORDS: Benzodiazepines; Immunoassay; Gas chromatography.

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INTRODUCTION

The presence of benzodiazepines is usually tested by screening urine samples by immunochemical techniques and confirming positive findings by GC [5], high-performance liquid chromatography (HPLC) [7, 12] or gas chromatography-mass spectrometry (GC/MS) [2, 6, 9, 12]. In many occasions it is necessary also to perform a quantitative benzodiazepine determination in the blood. This study compares benzodiazepine findings by GC and immunoassay techniques in blood and urine specimens for selecting a rational screening approach in post-mortem forensic toxicology.

MATERIALS AND METHODS

Sample preparation for gas chromatography and gas chromatography-mass spectrometry

Whole blood (1 g) was extracted with ethyl acetate (500 µl) at pH 7.4 [11]. Urine samples were hydrolysed by β-glucuronidase from *E. coli* K 12 (Boehringer Mannheim, Mannheim, Germany) and extracted using Isolute HCX SPE-columns (International Sorbent Technology Limited, Hengoed, UK) using an extraction procedure which was modified from Moore et al. [10]. Both blood and urine extracts were silylated with

N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) with 1% tert-butyldimethylsilyl chloride (TBDMSCl) [11]. Urine samples for GC/MS analysis were extracted using the same extraction procedure as for blood samples. The extracts were first acylated by pentafluoropropionic anhydride (PFPA) followed by silylation with MTBSTFA with 1% TBDMSCl [3].

Gas chromatography

Samples were analysed using two Micromat HRGC 412 dual-column gas chromatographs (HNU-Nordion, Helsinki, Finland) with DB-5 (15 m x 0.32 mm, 0.1μm film) and DB-17 (0.15 μm film) capillary columns and electron capture detectors under identical chromatographic conditions [11].

Gas chromatography-mass spectrometry

GC/MS was performed with a Hewlett-Packard (Wilmington, DE, USA) 5972 mass selective detector coupled to a HP 5890 Series II GC equipped with a HP-1 (12 m x 0.20 mm i.d. with 0.33 μm film) capillary column. The GC/MS was operated by Chemstation software and the GC was used in the splitless mode. The injector port temperature was 250°C and the transfer line temperature 280°C. The oven temperature was initially held at 130°C for 0.5 min and then increased by 15°C/min to 300°C, which was held for 2 min.

Data processing

Data processing was performed with MS Windows based SC-WorkStation 3.0 software (Sunicom, Helsinki, Finland). The programme was set to automatically identify the internal retention index (RI) standards by pattern recognition, to calculate the temperature programmed RI values of detected compounds, to compare them with library data, and to produce a combination report [11].

Immunoassay

Urine samples were analysed by Emit® d.a.u.™ Benzodiazepine Assay (ETSPLUS) (Syva Company, San Jose, CA, USA) with a cutoff of 200 ng/ml.

RESULTS AND DISCUSSION

For the present study, 506 such successive medical examiner's cases were selected where both urine and blood were available. Urine specimens without pretreatment were first analysed by immunoassay. Blood and enzyme-hydrolysed urine samples were screened for benzodiazepine drugs and metabolites by a dual-column GC method. The GC method allowed simultaneous quantitation of the drugs. Urine samples, which were negative or produced an invalid result by immunoassay but a positive result by GC, were also analysed by immunoassay after enzyme hydrolysis.

TABLE I. THE DETECTION LIMITS FOR BLOOD AND URINE

Compound	LOD (blood) [ng/g]	LOD (urine) [ng/ml]
Diazepam	10	10
Desmethyldiazepam	8	10
Oxazepam	5	5
Temazepam	5	5
Lorazepam	5	10
Chlordiazepoxide	50	–
Demoxepam	10	10
Clobazam	50	50
Norclobazam	20	30
Nitrazepam	10	5
Midazolam	30	–
1-Hydroxymidazolam	8	10
Alprazolam	20	40
1-Hydroxyalprazolam	5	10
Triazolam	3	–
1-Hydroxytriazolam	10	10
Clonazepam	10	10
7-Aminoclonazepam	20	[1]
Flunitrazepam[2]	3	5
Desmethylflunitrazepam	5	5
Phenazepam[3]	5	5
Lormetazepam[3]	5	5

[1] Extraction recovery too low, [2] Only in hospital use, [3] Not marketed in Finland.

Urine samples from those cases, where urine was negative by GC but either urine was positive by ETS or blood positive by GC, were analysed for amino metabolites by GC/MS using the same liquid-liquid extraction procedure and internal standard as with blood samples. The GC/MS analysis was performed by selected-ion monitoring, using three ions for each derivatized metabolite (7-aminoclonazepam: 602, 604, 659, 7-aminoflunitrazepam: 486, 487, 543 and 7-aminonitrazepam: 568, 569, 625) and internal standard (1,3-dihydro-1-ethyl-7-fluoro-5-(4-fluorophenyl)-2H-1,4-benzodiazepin-2-one: 299, 300, 271).

TABLE II. THE RESULTS OF THE COMPARISON STUDY

Material/method	A	B	C	D	E	F	G	H	I	J	K	Total positive
Urine (ETS)	–	+	–	–	+	–	+	+	invalid*	invalid	invalid	153/175**

Urine (GC)	–	+	+	+	+	–	–	–	–	+	+	200
Blood (GC)	–	+	+	–	–	+	–	+	–	–	+	185
											Total no. of cases
No. of cases	283	145	31	16	4	4	2	2	16	1	2	506

– Negative for benzodiazepines, + positive for benzodiazepines, *no result obtained by immunoassay.
** After enzyme hydrolysis of urine samples in groups C, D, J and K.

The GC detection limits of benzodiazepines in blood and urine are presented in Table I. The results of the comparison study are summarised in Table II. Chromatograms of an autopsy case with blood and urine extracts are presented in Figure 1.

The present study confirms the results reported by many previous authors [1, 4, 8]: enzymatic hydrolysis improves the detection limits of compounds forming glucuronide conjugates by immunoassay techniques (Tables III and IV), and it also seems that the

Fig. 1a. Dual-column chromatogram obtained from an autopsy blood sample. Findings: diazepam 0.24 μg/g, desmethyldiazepam 0.23 μg/g, oxazepam 0.02 μg/g, temazepam 0.08 μg/g and alprazolam 0.05 μg/g.

Fig. 1b. Dual-column chromatogram obtained from an autopsy urine sample. Findings: desmethyldiazepam 0.10 μg/ml, oxazepam 1.1 μg/ml, temazepam 2.1 μg/ml, alprazolam 0.10 μg/ml and 1-hydroxyalprazolam 0.19 μg/ml.

cutoff limit of 200 ng/ml is too high for many benzodiazepines. The negative blood results in Tables IV and V can simply be explained by the longer presence of metabolites in urine than their parent compound in the blood. The negative GC urine results in Tables VI, VII and VIII can be explained by a low extraction recovery of 7-aminoclonazepam in the present SPE procedure and the lack of an electron withdrawing group in 7-aminonitrazepam. In case no 2173 (Table VI), there was a recently given dose during resuscitation. There seems to be only one false positive by immunoassay (2130, Table VII).

TABLE III. GROUP C: URINE IMMUNOASSAY NEGATIVE, URINE GC POSITIVE, BLOOD GC POSITIVE

Case no.	Hydrolysed urine, GC [μg/ml]	Immunoassay (hydrolysed urine)

2006	Oxazepam 0.05	–
2012	Oxazepam 0.07, temazepam 0.08, diazepam 0.07, desmethyldiazepam 0.09	–
2056	Oxazepam 6	–
2082	Oxazepam 0.04, temazepam 0.9	–
2086	Oxazepam 0.1, temazepam 4.2	+
2088	Oxazepam 3.9	+
2091	Oxazepam 0.1, desmethyldiazepam 0.05	invalid
2094	Lorazepam 0.3	–
2119	Oxazepam 0.9	–
2133	Oxazepam 1.4	+
2124	Temazepam 0.5	–
2157	Oxazepam 0.04, temazepam 2	+
2166	Temazepam 0.4	–
2200	Oxazepam 0.6, temazepam 4.4	+
2276	Temazepam 1.7	+
2299	Oxazepam 0.07, temazepam 0.04	–
2369	Oxazepam 0.06, temazepam 0.06, desmethyldiazepam 0.03	+
2382	Oxazepam 0.2, temazepam 4	+
2401	Oxazepam 0.05, temazepam 0.2	–
2440	Oxazepam 0.07, temazepam 0.2, desmethyldiazepam 0.04	+
2487	Oxazepam 3.7	+
2489	Oxazepam 0.05, temazepam 0.09, desmethyldiazepam 0.06	+
2499	Oxazepam 5	+
2537	Temazepam 0.1	–
2580	Oxazepam 3.2	+
2586	Oxazepam 1.4	+
2590	Oxazepam 0.1, temazepam 0.7	+
2679	Temazepam 0.1, desmethyldiazepam 0.06, oxazepam 0.03	–
2781	Oxazepam 0.04, temazepam 0.04, desmethyldiazepam 0.05	+
2793	Temazepam 0.07, demoxepam 0.3, desmethyldiazepam 0.05	–
2815	Oxazepam 0.1, temazepam 0.5	–

TABLE IV. GROUP D: URINE IMMUNOASSAY NEGATIVE, URINE GC POSITIVE, BLOOD GC NEGATIVE

Case no.	Hydrolysed urine, GC [µg/ml]	Immunoassay (hydrolysed urine)
2027	Temazepam 0.05	–
2050	Lorazepam 0.6	+
2064	Oxazepam 0.1	–

2148	Demoxepam 0.3	–
2195	Oxazepam 0.05, temazepam 0.05	–
2214	Oxazepam 0.1, temazepam 0.06	+
2290	Demoxepam 0.3	–
2292	Temazepam 0.06	–
2336	Temazepam 1.5	+
2403	Temazepam 0.2	–
2415	Temazepam 0.07	–
2541	Oxazepam 0.1, temazepam 0.1, demoxepam 0.1	+
2605	Oxazepam 0.2	–
2723	Temazepam 0.4	+
2864	Demoxepam 0.3	–
2869	Temazepam 0.1	–

TABLE V. GROUP E: URINE IMMUNOASSAY POSITIVE, URINE GC POSITIVE, BLOOD GC NEGATIVE

Case no	Hydrolysed urine, GC [µg/ml]
2007	1-Hydroxymidazolam 0.4
2045	Demoxepam 0.8
2627	1-Hydroxyalprazolam 0.2
2854	Desmethyldiazepam 0.07, temazepam 0.05

TABLE VI. GROUP F: URINE IMMUNOASSAY NEGATIVE, URINE GC NEGATIVE, BLOOD GC POSITIVE

Case no	Blood, GC [µg/g]	Hydrolysed urine, GC/MS [µg/ml]
2173	Diazepam 0.03, desmethyldiazepam 0.03	–
2632	Clonazepam 0.1, 7-aminoclonazepam 0.8	7-Aminoclonazepam 0.08
2798	7-Aminoclonazepam 0.3	7-Aminoclonazepam 0.06
2842	7-Aminoclonazepam 0.03	7-Aminoclonazepam 0.05

In post mortem forensic toxicology, the present GC method for blood seems to be a good alternative to the common combination of urine immunoassay followed by quantitative analysis of blood by chromatography. A disadvantage of the present GC method is the fact that the amino groups of the 7-amino metabolites of clonazepam and flunitrazepam do not silylate in the present silylation procedure, and consequently the detection limits of these metabolites are high. 7-Aminoclonazepam was included but 7-aminoflunitrazepam was excluded from the screening. However, there was only one case which was found positive for an amino metabolite (2515, Table VII) without the parent compound detected in the blood. The influence of flunitrazepam use is not shown in the present study because flunitrazepam is only in hospital use in Finland.

TABLE VII. GROUP G: URINE IMMUNOASSAY POSITIVE, URINE GC NEGATIVE, BLOOD GC NEGATIVE

Case no	Hydrolysed urine, GC/MS [µg/ml]
2130	–
2515	7-Aminonitrazepam 0.6

TABLE VIII. GROUP H: URINE IMMUNOASSAY POSITIVE, URINE GC NEGATIVE, BLOOD GC POSITIVE

Case no	Blood, GC [µg/g]	Hydrolysed urine, GC/MS [µg/ml]
2547	7-Aminoclonazepam 0.8	7-aminoclonazepam 0.1
2551	Nitrazepam 0.02	7-aminonitrazepam 0.2

The results also reveal the prevalence of benzodiazepines: 40.7% of the post mortem cases received for drug screening were positive for benzodiazepines.

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