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Quantitation by gas chromatography with selected-ion monitoring mass spectrometry of "natural" diazepam, N-desmethyldiazepam and oxazepam in normal human serum

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ABSTRACT

During the past five years, the literature has tended to prove the occurrence of "natural benzodiazepines" in tissues and biological fluids of non-medicated humans. Several have been identified but very few papers deal with their quantitation in biological material. We present here a method for the specific and sensitive measurement of serum levels of diazepam, N-desmethyldiazepam and oxazepam by gas chromatography with selected-ion monitoring mass spectrometry in twenty human volunteers without medication. Diazepam was found over the whole population, in the range 7.3–32.0 pg/ml, identical in males and females. The other two were present in only some individuals (1.0–7.6 pg/ml for N-desmethyldiazepam and 2.0–13.0 pg/ml for oxazepam). The origin (endogenous, dietary or microbial) of these substances is still to be elucidated.

INTRODUCTION

The fortuitous discovery of the pharmacological properties of benzodiazepines (BZDs) was made in 1957 [1]. In 1977, high affinity binding sites for BZDs, whose binding kinetics parrallel their pharmacological activities, were identified in cerebral cellular membranes [2,3], and consequently were called receptors; proof was given of their coupling to γ-aminobutyric acid (GABA) receptors 4], GABA being the main inhibitory neurotransmitter in the brain. Other BZD binding sites, not coupled to GABA sites, have been identified at the periphery, and also in glial cells in the brain [5].

The presence of these receptors suggested the hypothesis that natural molecules similar to BZDs could behave as natural ligands for these sites; the same kind of argument previously led to the discovery of endorphins after the characterization of morphinic cerebral receptors. The search for natural ligands for these sites led to various compounds (proteins, puric bases, nucleotides); however, most of them are no longer tak-

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en into consideration because they have weak affinities or specificities for the sites [6]. On the contrary, the recent discovery of "endogenous" BZDs, after some polemic discussions, is now well documented, although their "endogenous" origin has not yet been proven.

Five years ago, Sangameswaran (see review in ref. 7) isolated from beef brain and cerebellum, by immunoaffinity chromatography and high-performance liquid chromatography (HPLC), two benzodiazepinic molecules identified as N-desmethyldiazepam and oxazepam. Pena *et al.* [8] recently detected a benzodiazepine-like immunoreactivity in the plasma of human subjects. In 1988, De Robertis *et al.* [7] expanded several hypotheses concerning the eventual origin of "endogenous" BZDs: either contamination during analytical procedures, or contamination resulting from industrial synthesis, but these arguments were rendered unlikely by the discovery of BZDs in the cerebral tissues of people who died in 1940, before the production of synthetic BZDs [9]. A third hypothesis speculated on an alimentary origin of BZD compounds: cow milk [10] and several vegetables and cereals [11] contain BZDs, as well as some penicilliums and the intestinal flora.

Because of the numerous questions concerning the investigation of these molecules in human samples, and their interpretation, it seemed to us useful to further investigate three BZD molecules (Fig. 1) in the blood of human subjects, using the sensitive and specific gas chromatography (GC) with selected-ion monitoring (SIM) mass spectrometry (MS), with an internal standard.

EXPERIMENTAL

Subjects

Twenty healthy volunteers, eleven female and nine male (24–43 years) participated by supplying blood samples under medical control. The subjects had been informed about the nature of the test and the local ethical committee had approved the project. Sera from 50-ml blood samples collected in polypropylene vials were separated and stored at −20°C.

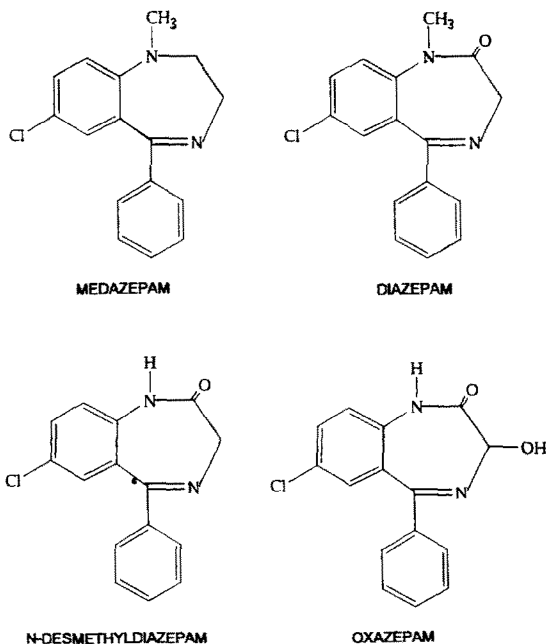

Fig. 1. Molecular structures of some benzodiazepines.

Reagents and chemicals

Diazepam (DZ), N-desmethyldiazepam (DD), oxazepam (OX) and the internal standard, medazepam (MD), were purchased from Roche (Neuilly sur Seine, France). Drug standard solutions (100 mg/l) were prepared in ethyl acetate and stored at 4°C in the dark. N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA). Toluene, ethyl acetate and sodium hydroxide were supplied from Merck (Darmstadt, Germany). The "drug-free" serum Lyphocheck was purchased from Biorad (Anaheim, CA, USA). All pH values were verified with a Tacussel pH meter (Villeurbanne, France).

Instrumentation

The analysis was carried out on a gas chromatograph–mass spectrometer QP 1000 (Shimadzu, Tokyo, Japan) with an automatic sample injector (AOC-9, Shimadzu). The computer for acquisition and processing of data was a V286 (Victor Technologies, Stockholm, Sweden), connected to a printer KX-P 1083 (Panasonic, Matsushita Electric Trading, Osaka, Japan). The

wide-bore borosilicate glass column was an SPB-1 (30 m × 0.75 mm I.D., 1.0 μm film thickness) from Supelco (Bellefonte, PA, USA); the carrier gas was helium, N60 (Air Liquide, Paris, France); the column head presure was 0.20 bar; the splitless injector temperature was 280°C. The GC oven temperature was initially 230°C for 2 min, then programmed at 7°C/min to 280°C, then held for 2 min; the total cycle time was 10 min. Electron impact (EI) ionization with 70 eV energy and an emission current of 60 μA were used; the temperature of the transfer line and the jet separator was 280°C, and that of the ion source 250°C. The make-up gas flow-rate was 20 ml/min. The data were obtained in scan mode from 49 to 750 atomic mass units (amu) at 1.4 s per cycle, or in SIM mode. The instrument was autotuned with perfluorotributylamine (PFTBA). The electron multiplier voltage was 1400 V in scan mode and 3200 V in SIM mode. The masses monitored were: for medazepam, m/z 242; for diazepam, m/z 256; for N-desmethyldiazepam, m/z 327, and for oxazepam, m/z 458.

Sample preparation

After thawing, 20 ml of each serum was spiked with 0.5 ng of MD, then alkanized to pH 9 (checked with a pH meter) with sodium hydroxide (45%). A double extraction was carried out by adding two 20-ml volumes of toluene–ethyl acetate (80:20), with slow agitation, during 10 min. Then the organic phase was separated by centrifugation for 10 min at 2300 g and evaporated under a stream of nitrogen at room temperature.

Derivatization

OX and DD can be derivatized by silylation reagents; silyl derivatives are formed by the exchange of the active hydrogens from amines or alcohols by the trimethylsilyl group. DZ and MD cannot be derivatized (no active hydrogens). The organic extract (20 ml) was evaporated at room temperature under a stream of nitrogen. The dry residue was redissolved in 100 μl of MTBSTFA, vortex-mixed to aid dissolution, then sonicated and finally incubated at room temperature for 1 h. A 5-μl volume was injected into the gas chromatograph.

Calibration curves and quantification

A calibration curve for DZ, DD and OX in the range 2.5–50 pg/ml serum (2.5, 5.0, 10.0, 25.0 and 50.0 pg/ml) was obtained with working solutions diluted in the above solvent, by spiking 20-ml aliquots of a drug-free serum with a mixture of all three drugs. The drug-free serum was checked for the absence of DZ, DD, OX and MD by GC–SIM-MS. MD (25 pg/ml serum) was added to each tube before extraction. Each calibration point (n = 2) was extracted and derivatized according to the above procedure. The calibration curves served to quantify DZ, DD and OX in sera.

Quality control

For quality control of the method, standard curves were established using drug-free serum samples spiked with mixtures of DZ, DD and OX. Each curve was drawn using three levels of concentration (low, 2.5 pg/ml; medium, 10 pg/ml; high, 50 pg/ml). Three assays were done for each level, by performing three extractions every day during three consecutive days. The coefficients of variation (C.V.) obtained for within-run precision evaluation of the three levels of concentration were: 18%, 2.5% and 2% for DZ; 15%, 2.5%, 1.5% for DD; and 10%, 2%, 1.5% for OX. For between-run precision, the C.V. values were: 21%, 3%, 2.5% for DZ; 17%, 2.5% and 2% for DD; and 15%, 2% and 1.5% for OX. Data in Table I show good linearity (r = 0.999) in the 2.5–50 pg/ml range for DZ, DD and OX. The limit of detection (LOD = $4x$) was calculated by running ten reagent blanks and taking the mean value x: the LOD can be chosen at 2 pg/ml (experimental value 1.84 pg/ml) for DZ, at 0.5 pg/ml (experimental value 0.36) for DD and 1 pg/ml (experimental value 0.84) for OX. Concerning the limit of quantification (LOQ = $6x$) calculated in the same manner, the values found were: 3 pg/ml for DZ (real 2.80 pg/ml), 1 pg/ml for DD (real 0.55) and 1.5 pg/ml (experimental value 1.27) for OX.

TABLE I

LINEAR REGRESSION OF DIAZEPAM, N-DESMETHYLDIAZEPAM AND OXAZEPAM

Linear regression parameters $y = bx + a$; $n = 3$.

Compound	Concentration range (pg/ml)	Slope, b	y-intercept, a	Correlation coefficient, r
DZ	2.5–50	0.0381	−0.0239	0.9997
DD	2.5–50	0.2291	0.1298	0.9996
OX	2.5–50	0.0386	0.0202	0.9990

We have also checked the "drug-free" serum from Biorad for the presence of trace amounts of BZDs. Only when 40-ml samples were run throughout the procedure, were traces of DD and OX detectable, beyond LOQ values. For the 20-ml standard volume used in all assays no signal appeared for all three BZDs.

The stability of drug solutions in ethyl acetate stored at 4°C proved to be excellent over a six-month period; this was not the case for derivatized BZDs, which were stable only for 48 h.

RESULTS AND DISCUSSION

Confirmation of identity

In view of the very low concentrations found in sera, mass spectra could not be obtained because of the low sensitivity of the TIC mode. For this reason, tentative confirmation of identity was done only by SIM, by measuring retention times on the more polar column SP-2250. Unfortunately, even with this intermediate polarity column, separation of BZDs remained impossible. For this reason, we decided to confirm identification on the same column, changing only the temperature programme: initial temperature, 235°C for 4 min, then programmed at 14°C/min to 280°C, then held for 2 min. Retention times for the first programme were 2.40 min for MD, 3.49 min for DZ, 4.20 min for DD and 6.28 min for OX. For the confirmation programme, they were 2.65 min for MD, 4.22 min for DZ, 4.92 min for DD and 7.13 min for OX. All serum extracts showed an exact correspondence in retention times with both temperature programmes.

We also decided to carry out a second confirmation by performing SIM on two characteristic fragments of each BZD: ions m/z 458 and 242 for OX; ions m/z 327 and 242 for DD; ions m/z 256 and 242 for DZ. Fig. 2A shows fragmentograms obtained by SIM on a serum extract from a 25 pg/ml calibration point (identical concentration for MD and all three drugs in the mixture). Fig. 2B is an example of SIM fragmentograms obtained from the serum of a female subject. The fragmentogram from ion 242 shows the presence of four peaks at the following retention times: 2.40 min (MD), 3.49 min (DZ), 4.20 min (DD) and 6.28 min (OX). These are values identical with those obtained with the second characteristic ion of each BZD: 2.40 min (ion 242), 3.48 min (ion 256), 4.19 min (ion 327), and 6.27 min (ion 458). Some peaks correspond to ions of low intensity for a given BZD so that the signal had to be amplified: the amplification factor (4, 32 or 64) is indicated on the figure.

So, we can conclude that the above results show unambiguous absolute identification, because it is highly improbable to find at a given retention time a substance identified by a couple of characteristic ions.

The correlation between the peak-area ratio of ions m/z 256/242 (DZ), 327/242 (DD) and 458/242 (OX) and concentration in pg/ml was excellent, as indicated in Table I.

Table II gives the results obtained for both populations. DZ mean values were: 15.83 pg/ml in men; 15.89 pg/ml in women; 15.86 pg/ml for the whole population. For DD and OX, individual results show that some subjects had values above the LOQ; others were under this limit although, from a qualitative viewpoint, the presence of BZD was still certain.

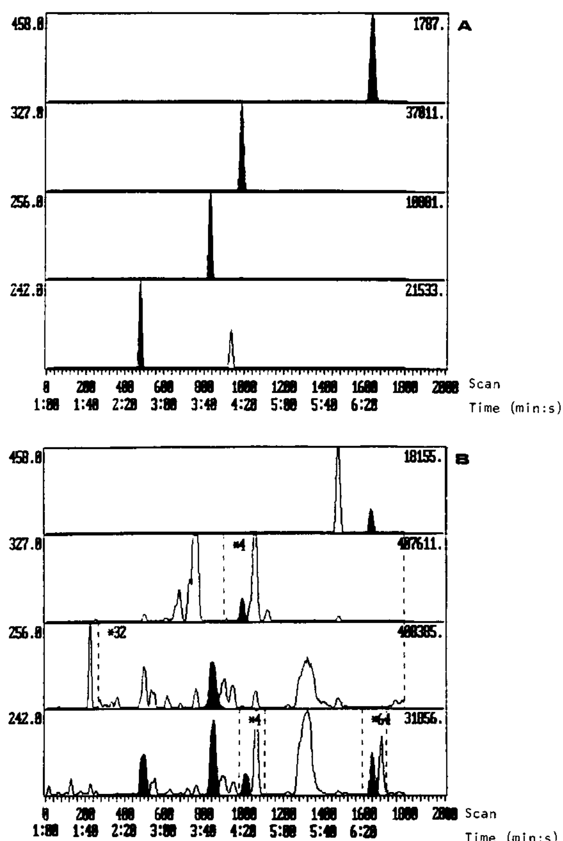

Fig. 2. Selected-ion chromatograms of serum extracts for (A) a calibration curve point at 25 pg/ml and (B) a human subject. The four numbers on the *y*-axis correspond to the four SIM fragmentograms obtained with each characteristic ion.

The ANOVA test, applied to comparison of male and female populations for DZ, has given no difference ($F_{0.975} = 0.00$).

The characterization of BZD receptors in 1977 suggested the existence of endogenous ligands for these binding sites and, among several candidate molecules, BZDs themselves (see reviews in refs. 7 and 11). The identification of such compounds in human blood, cerebrospinal fluid and tissues, mainly brain and liver, opened discussions first on methodological procedures (primary investigations were immunological) then on their origin: alimentary or synthesis by intestinal flora, or endogenous. Synthesis of such molecules has not yet been demonstrated in mammalian tissues *in vitro*; however, BZD-like immunoreactivity has been detected in cultures of neuroblastoma × glioma cells monitored for three months in a germ-free medium. In fact, the ability of BZD binding sites to bind molecules with either agonist or inverse agonist properties make it possible that these sites could be regulated by two endogenous ligands with opposing actions (see review in refs. 7 and 11).

The presence of BZD molecules in human samples has been demonstrated by six independent groups (including us) using different evaluation procedures: immunological (histology [9,12]), radioimmunoassay (RIA) [8,13], radioreceptor assay (RRA) [12–15], GC with electron-capture detection [16] and GC–MS. So it is very unlikely that all these findings are wrong, although the quantitative evaluations differ probably owing to methodological reasons.

Since the levels of BZDs likely to be found in human sera are very low, our purpose was to choose a procedure providing specificity and avoiding any interference. We used GC–SIM-MS for its specificity and sensitivity, together with the absence of cross-reactivity, which is often observed with some of the other techniques quoted above.

We got assurances that the human subjects had not received any BZDs for the previous six months. During collection of samples and purification steps, polypropylene vials were used: glassware is unsuitable because it adsorbs very small amounts of BZDs. To avoid contamination of the syringe and every material, a ten-fold cleaning was done with the solvent.

Our choice of which BZD to investigate was made because "natural" diazepam and N-desmethyldiazepam had already been found by several authors in human samples. Oxazepam is a metabolic compound resulting from DD oxidation. Likewise, DD is a metabolite of a great number of BZDs especially DZ. The internal standard (MD) was chosen because of its analogous structure and chromatographic behaviour. The differences between the retention times are sufficient to produce chromatographic separation over a 10-min time range.

The determination of BZDs in serum or plasma has been rarely reported in the literature.

TABLE II

SERUM CONCENTRATIONS OF DIAZEPAM, N-DESMETHYLDIAZEPAM, OXAZEPAM AND DIAZEPAM EQUIVALENT (DE) (TWENTY HUMAN SUBJECTS), QUANTIFIED BY GC–SIM-MS

Male					Female				
Subject	Concentration (pg/ml)				Subject	Concentration (pg/ml)			
	DZ	DD	OX	DE		DZ	DD	OX	DE
1	16.4	3.9	4.4	24.7	1	11.6	7.6	9.2	28.4
2	17.6	5.5	4.7	27.8	2	C[a]	2.8	+[b]	
3	32.0	3.5	2.0	37.5	3	21.6	0[c]	+	21.6
4	12.5	2.1	+	14.6	4	10.8	1.8	+	12.6
5	12.1	+	+	12.1	5	24.6	0	0	24.6
6	13.7	+	+	13.7	6	27.4	1.0	+	28.4
7	17.2	2.2	+	19.4	7	16.8	1.1	+	17.9
8	8.1	0	+	8.1	8	13.1	0	+	13.1
9	12.9	0	+	12.9	9	14.0	2.3	+	16.3
					10	7.3	0	+	7.3
					11	12.2	6.2	13.0	31.4
$\bar{x}$	15.83 ± 2.25[d]				$\bar{x}$	15.89 ± 2.05[d]			

[a] C = Contamination.
[b] + = Traces below detection limit (DZ, 2.0 pg/ml; DD, 0.5 pg/ml; OX, 1.0 pg/ml).
[c] 0 = Not detectable.
[d] $\bar{x}$ = Mean ± standard error of the mean in pg/ml.

Wildmann *et al.* [15] used RRA in rat plasma to quantify three BZDs at the level of 10–25 ng/g, expressed in DZ equivalent. Unseld *et al.* [14] studied four human subjects: DZ was not identified (detection limit 5 pg/ml plasma) and DD was found between 3 and 10 pg/ml. Olasma *et al.* [13] recently used RIA and RRA to analyse BZDs in the sera of fifteen normal human subjects, and found levels of 1.3 ± 0.5 pmol/ml, expressed in DZ equivalent (370 pg/ml of "diazepam"). The RRA shows that rats have BZD levels far higher than humans (*ca.* hundred-fold). GC–MS results are more specific: only DD could be detected and quantified; DZ levels were too low to be quantified.

Our results were obtained from twenty human subjects. This allowed us to establish for the first time a valuable statistical study.

Levels of DD are in the range 1.0–7.6 pg/ml; some individuals were under the detection limit (1.0 pg/ml) and in others DD was undetectable. Our range is very near of that determined by Unseld *et al.* [14] (3–10 pg/ml). Levels of OX are in the range 2.0–13.0 pg/ml, and again some individuals were below the detection limit. No references were found in the literature. For DZ, the range is 7.3–32.0 pg/ml, and female and male populations are statistically equivalent (x = 15.89 ± 2.05 and 15.83 ± 2.2 pg/ml respectively). If we consider the sum of the three BZDs (see Table I), expressed in DZ equivalent (DE), comparisons become possible with the RRA results of Olasma *et al.* [13] This comparison shows that BZD levels found by RRA are approximately ten times higher than those found by GC–MS. Of course, this comparison is only approximate if we consider other "natural" BZDs or metabolites not quantified by us. We consider it unlikely that this could explain the ten-fold difference found.

CONCLUSION

Our results clearly demonstrate the presence of "natural" BZDs, especially diazepam, in human

serum. Diazepam has not been quantified previously. Its mean level was found to be *ca.* 16 pg/ml serum. Other BZDs are less consistently found. Current work in our laboratory will try to elucidate the origin (endogenous, dietary or microbiological) of these molecules.

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