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QUANTITATION OF BARBITURATES IN URINE BY GC/MS AND ITS COMPARISON TO FLUORESCENCE POLARIZATION IMMUNOASSAY

Hea-Young P. Choo*, Jeongeun Choi, Myung Ja Choi,
Eun Young Song+ and Jongsei Park**

Doping Control Center Korean Institute of Science and Technology, Seoul 136-791
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ABSTRACT: *Barbiturates are commonly abused tranquilizer and a rapid method to determine these drugs in biological samples is needed. In this study, we screened barbiturates in urine specimens by the fluorescence polarization immunoassay method(FPIA) and the positive samples were confirmed and identified by the more definitive GC/MS method. Fifteen positive samples which have barbiturate values higher than 0.5 μg/ml were analyzed by the GC/MS method. Eight samples were identified as phenobarbital and five samples were identified as crotilbarbitone. Phenobarbital showed the peak at 4.0 min retention time with the characteristic peaks of m/z 232, 117, 175 and 260 after methylation. Crotilbarbitone showed strong peak of the characteristic ions m/z 212, 181, 156, 55 and 141 without methylation. When comparing the phenobarbital values obtained by the FPIA and the GC/MS method, the FPIA method generates higher values than the GC/MS method. The results suggest that the FPIA method could determine both phenobarbital and the structurally similar phenobarbital metabolites together.*
Key words: *Barbiturate assay, GC/MS, Fluorescence polarization immunoassay.*

INTRODUCTION

The classical approach to measurement of barbiturates in biological specimens involves ultraviolet spectrophotometry (Goldbaum, 1952). Gas chromatography provides a more specific result and is usually performed with flame-ionization or

*Current address: Ewha Womans University, School of Pharmacy, Seoul, Korea
+ Current address: Genetic Engineering Research Institute, KIST, Seoul, Korea
**Author to whom requests for reprints should be addressed.

nitrogen-selective detection of the free drugs (Prorchik, 1975; Ritz and Warren, 1975; Killen and Petters, 1983). Recently, electron capture detector and mass spectrometer are also employed as GC detectors for higher sensitivity (Dill and Pillai, 1980; Sun and Chun, 1977; Skinner *et al.*, 1973; Patel *et al.*, 1980). Phenobarbital measurement has been included in a general liquid chromatographic scheme for common anticonvulsant drugs (Kabra and McDonald, 1978).

Chromatographic assay provides more specific information, i.e., it can separate a parent drug from its metabolite(s), more accourate information in quantitative measurements and assay method can be customized depending on a lab.'s need and operating costs are low. Nevertheless, it requires higher initial investment and it also needs highly trained personnel not only for operation but also for data interpretation.

Immunoassy in drug assay can provide quick results (in general, within one minute compared to 5 to 20 minutes by chromatographic assay for one sample) and is very suitable for screening large numbers of specimens in a short time period. In many cases immunoassay provides more sensitive data than the GC/MS method, but it lacks specificity depending on the assay reagent's cross-reactivity. Also, a laboratory can perform assay(ies) only with commercial regents. Because of the advantages and disadvantages of each method, it is desirable to use immunoassay as a screening tool and to use GC/MS assay as a confirmation tool for presumptive positive samples by immunoassay when a laboratory is required to test few hundred samples within a day. Abbott Laboratories (Chicago, U.S.A.) recently introduced its abused drug assays using the fluorescence polarization immunoassay (FPIA) method for testing urine specimens with the TDx analyzer.

In this study, the results of the FPIA method were compared with the results of the more definitive GC/MS method in the barbiturate assay. This was because it is always useful to compare a new procedure with established or reference methodology for the evaluation of any new testing procedure. Prior to testing the specimens, the linearity and precision of the FPIA method was established. Barbiturates in specimens that gave positive results by FPIA were confirmed and identified by GC/MS and the values obtained by both methods were compared.

MATERIALS AND METHODS

Reagents

Phenobarbital and hexobarbital were obtained from Deutsche Sporthochschule Koln Institute fur Biochemie (Koln, Germany). Other reagents were of analytical grade and were used without further purification.

Equipment

1. The TDx analyzer was equipped with Revision 11.2 software and the following reagents were obtained from Abbott Labs. (Chicago, USA).
   Calibrators (Secobarbital): 6 levels
   Controls: 2 levels
   Bar-coded reagent pack:
   Drug antiserum: sheep antiserum in buffer.

Fluorescein tracer(F*): Fluorescein labeled drug.
Pretreatment solution: Protein and NaN_3 in buffer.
Buffer (wash solution): Protein and NaN_3 in buffer.

2. The gas chromatograph was the Hewlett-Packard 5890 equipped with a 5970 mass selective detector. The column was a cross-linked 5% phenylmethyl silicone capillary (length 16 m, I.D. 0.2 mm. film thickness 0.33 μm). Injector temperature was 280℃ and samples were injected in split mode (split ratio, 1:10). Oven temperature was started at 150℃ and ramped 15℃/min to 300℃ and held for 3 min. The transferline temperature was 300℃. Helium at the flow rate of 0.8 m*l*/min was used as a carrier gas. Selected ion monitored for N,N'-dimethyl phenobarbital were 232, 117 and 260 whereas 235, 169 and 250 were used for N,N'-dimethylhexobarbital. Ions 232 and 235 were used for quantitation only.

Calibration Curve for GC/MS

Methanolic solution of phenobarbital was spiked into blank urine to give concentrations of 0.1, 0.2, 0.5, 1.0 and 2.5 μg/m*l* respectively. The internal standard hexobarbital was added to give a concentration of 0.8 μg/m*l*.

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Extraction and Methylation of Phenobarbital in Urine for GC/MS

The pH of a 5 m*l* urine sample was adjusted to 1-2 with 6 N-HCl. Ether (5 m*l*) was added to the sample and the mixture was agitated mechanically for 20 minutes. Two layers were separated by centrifugation (2500 rpm, 5 min). The etheral phase was transferred into another test tube, and the solvent was evaporated in a rotary evaporator. The sample was dried in a desiccator over P_2O_5/KOH. The dried residue was redissolved in a mixture of acetone (200 μ*l*), methyl iodide (20 μ*l*), and potassium carbonate (100 mg). The mixture was sealed with a glass stopper and heated to 60℃ for 30 min. The methylated sample (1 μ*l*) was analyzed in GC/MS.

TDx Procedure

The samples were clarified by centrifugation at 2000 rpm for 10 min. 100 μL of the samples was applied to the sample cup in the carousel. Prior to the assay, the analyzers were calibrated by determining the polarization intensity of the pipet check solution. The drug assay parameters were stored according to the manual and the standard curve of barbiturate was established using 6 levels of calibrator solution. The validate the assay values in the sample, two levels of controls were included in each run of the assay.

The serial dilution of a urine sample with high drug concentration gave a linear response with greater than 0.99 of correlation coefficients. Therefore, a high concentration sample was diluted with a buffer and a sample value was obtained.

RESULTS AND DISCUSSION

It would be an ideal situation if an assay method that could screen and confirm the presence of drugs in a biological specimen, within a short turnaround time was available. The TDx system of Abbott Laboratories was used in conjunction with gas chromatography/mass spectrometry for the measurement of phenobarbital levels the in urine. The TDx system is a homogeneous competitive immunoassay system utilizing fluorescence polarization immunoassay methodology and computerized automation with bar-coded reagents. The assay provided a direct measure of bound and free tracer without a separation step. The principle of TDx commpetitive FPIA is described elsewhere (Jolley *et al.*, 1981).

The TDx results were evaluated as semiquantitative because the immunoassay detected the parent drug and major metabolites together and antibodies in it cross-reacted to drug molecules. The specificity data in the system indicated that many drugs of similar chemical structures cross-reacted in high degrees. Therefore, high values of the TDx screen results were subjected to confirmation and identification for individual drugs by gas chromatography/mass spectrometry which has high specificity. Because the TDx system does not require sample pretreatment steps and provides quick results, these advantages over GC/MS make the system more useful for screening samples semiquantitatively.

Within-run precision of the TDx assay was tested at 2 concentration levels with ten replicates. Coefficients of variation ranged from 1.6-3.7% with an overall CV of 2.7%. Between-run precision was tested at 2 concentration levels 33 times of consecutive analytical runs. Coefficients of variation ranged from 3.1-5.3% with an overall cv of 4.2% (Table 1).

Table 1. Precision study by TDx barbiturate assay

	Conc. (mg/l)	N	% CV
Within-Run	1.0	10	3.7
	0.6	10	1.6
Between-Run	1.0	33	5.3
	0.6	33	3.1

Calibration of Phenobarbital for GC/MS

Fig. 1 showes the full mass spectra of phenobarbital and hexobarbital after methylation. When the samples were analyzed in selected ion monitoring of ion 232 and 235, the calibration curve calculated by the area ratio of the two peaks showed the good linearity (Fig. 2; $r=0.999$, $n=3$).

Quantitation of Phenobarbital for GC/MS

The amount of phenobarbital spiked into urine was analyzed by GC/MS and TDx and the result is presented in Table 2. In low concentration, both GC/MS and TDx gave a higher value than the actually added value. In high concentration, the TDx showed the higher deviation.

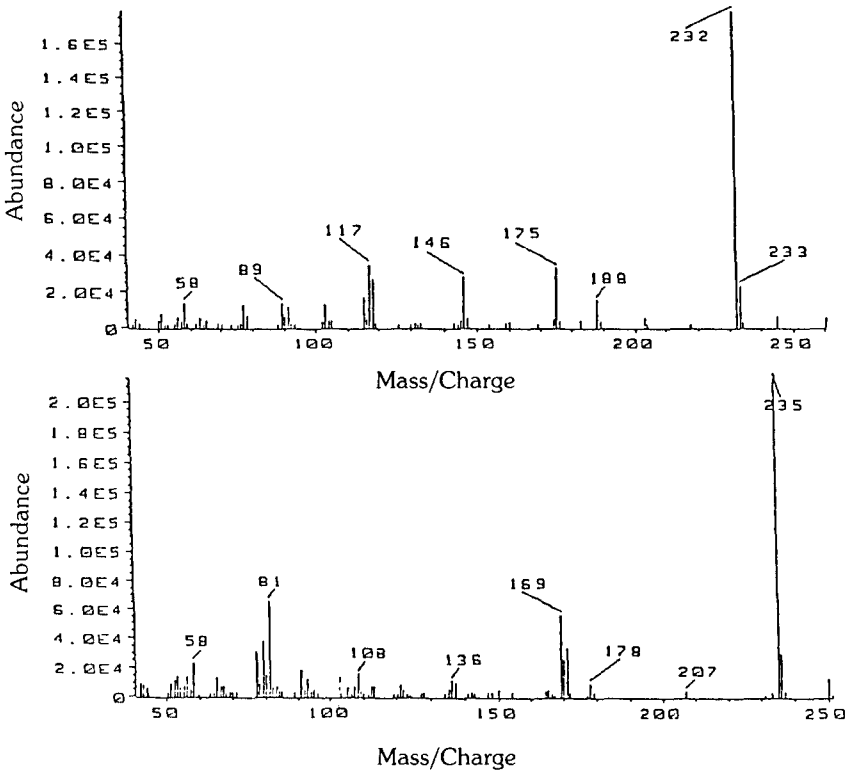

Fig. 1. Mass spectra of phenobarbital (A) and hexobarbital (B) after methylation.

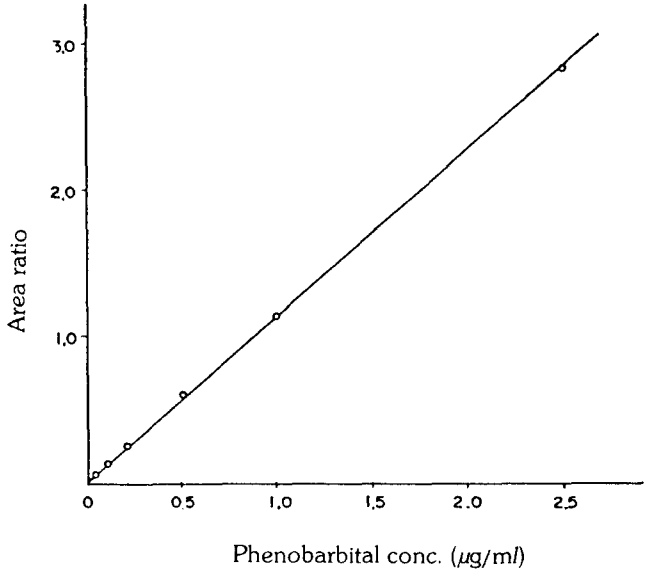

Fig. 2. Calibration curve for the quantitation of phenobarbital by GC/MS.

Comparison of FPLA Positive Results with GC/MS Results

Fifteen samples whose TDx barbiturate assay results were higher than 0.5 μg/ml, were analyzed by the GC/MS method described above. Eight samples were indentified as phenobarbital as shown in Table 3. Phenobarbital showed the peak at 4.0 min retention time, with the characteristic peaks of *m/z* 232, 117, 175 and 260 after methylation. Again by GC/MS, five samples showed strong peaks of the characteristic ions *m/z* 212, 181, 156, 55 and 141 without methylation. These ions are matched to the mass spectrum of crotilbarbitone. Eight phenobarbital positive cases were analyzed by both methods, and the results are presented in Table 3. Here the higher values for TDx were expected since TDx can determine the structurally similar phenobarbital metabolites. Therefore, all the eight cases sowed lower values for GC/MS analysis than for TDx analysis.

Table 2. Quantitation of phenobarbital in spiked urine by GC/MS and TDx

Amounts added (μg/ml)	Amounts found (μg/ml)(n=3)	
	GM/MS	TDx
0.05	0.024±4.0	0.091± 9.1
0.1	0.130±4.6	0.143±10.4
0.2	0.272±4.8	0.263± 4.2
0.4	0.458±3.3	0.522± 4.6
0.5	0.637±4.3	0.700± 2.3
1.0	1.156±3.6	High*
1.4	1.454±4.9	High
2.5	2.835±2.2	High

*High=over 2 μg/ml

Table 3. Concentration of barbiturates as a group by TDx and its identification by GC/MS in urine specimens

Sample number	Phenobarbital (μg/ml) GC/MS	Barbiturates (μg/ml) TDx
1	0.34	1.42
2	0.39	0.47
3	0.27	0.90
4	0.40	1.20
5	0.83	2.26
6	0.21	0.66
7	0.33	0.54
8	0.05	0.20
9	N.B.1	2.35
10	〃	1.66
11	〃	1.53
12	〃	0.89
13	〃	0.81

N.B.1.; These samples were identified to contain barbituraturates as crotilbarbitone by GC/MS assay.

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