Arch. Pharm. Res. 9(3), 131-138 (1986)

Determination of Barbiturates in Plasma by Gas Chromatography-Flame Photometric Detector after N,N'-Dimethylthiomethyl Derivatization

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(Received June 25, 1986)

Abstract □ A specific and sensitive gas chromatographic (GC) procedure with the flame photometric detector (FPD) was developed for determination of barbiturates such as barbital, allobarbital, secobarbital, phenobarbital and thiopental in plasma. In order to evaluate the performance of the FPD, the results were campared with those of the flame ionization detector (FID).

After extraction of barbiturates from plasma, the barbiturates were quantitatively N,N'-dimethylthiomethyl (MTM)-derivatized with methylthiomethyl chloride in 1,8-diazabicyclo [5.4.0] undec-7-ene catalyst.

The data indicate that the FPD is about 4 times more sensitive than the FID for barbiturates, although it is less reproducible. The FPD also produced chromatogram with less background for extracted plasma sample. The minimum detectable amount of MTM-thiopental on 3% OV-225 column was 4.4fmol and that of other MTM-barbiturate was about 10.0fmol.

Keywords □ Barbiturates, N,N'-Dimethylthiomethyl derivatization, Gas chromatography-flame photometric detection.

Several authors have investigated the GC methods of barbiturates which are used as sedative, hyphotics and anticonvulsants. The GC methods involving underivatized barbiturates (1-3) are clearly limited by column adsorption, so polarity of the free acid shoud be reduced by derivatization methods of barbiturates.

Derivatization methods of barbiturates reported by many authors are as following: (a) alkylation with dialkyl sulfate (4-6), alkyl iodide (7, 8) and diazomethane (9), (b) flash alkylation with tetraalkylammonium hydroxide (10-12), (c) benzylation (13), (d) acylation (14), (e) dimethoxylation (15) and (f) silylation (16). These derivatives were detected by the FID (3, 4, 7, 8, 12, 16), the electron-capture detector (6, 13, 14), the photoionization detector (1), the nitrogen-phosphorus sensitive detector (2), the hydrogen flame detector (5) and the mass spectrometer (9-11) and so on.

This paper describes a new GC method for the quantitation of barbiturates in rat plasma after MTM-derivatization. It is sensitive to about 4.4-10.0 fmol/injection using the FPD.

EXPERIMENTAL METHODS

Reagents and Materials

Allobarbital (Tokyo Kasei, Japan), sodium barbital (E. Merck, W. Germany), sodium phenobarbital (E. Merck), sodium secobarbital (Sigma, U.S.A) and sodium thiopental (KP IV) were used as supplied and 100 nmol/ml of heptadecanoic acid methyl ester (HAME) was prepared by esterification of heptadecanoic acid (Sigma) with borontrifluoride-methanol complex (E. Merck).

Hexamethylphosphorictriamide (HMPA, Wako, Japan) was distilled from calcium hydride (Aldrich, U.S.A) and methylthiomethyl chloride (Aldrich) and dimethylsulfoxide-d6 (DMSO-d6, E. Merck) were used without further purification.

Kieselgel 60HF254 and the pre-coated plate with Kieselgel 60F254 were obtained from E. Merck. Kieselgel 60F254 was washed with a mixture of methanol and chloroform (1 : 2) and then activated at 110 ℃ for 4 hours. All chemicals were used as guaranteed or spectroscopic reagent grade.

Blood was obtained from male albino Wistar rats

weighing 300-320g, and was centrifuged to seperate the plasma.

Gas Chromatography

A gas chromatograph (Pye-Unicam model 104, England) equipped with a sulfur-specific FPD and an FID was used. A 0.2m×0.4cm i.d. coiled glass column was packed with 3% OV-225 coated on acid-washed, silanized Gas Chrom Q (100-120 mesh) or with 3% OV-1 on Chromosorb WAW -DMCS (80-100 mesh). Column temperature was initially kept at 200 ℃ for 3min and then programmed to 260℃ at 5℃/min. Temperatures of injection port and detector were 280℃ and nitrogen carrier gas flow rate was 30ml/min; flow rates of hydrogen and air were 35ml/min and 27 ml/min in the FPD and were 20 and 100ml/min in the FID, respectively; the amplifier sensitivity range was set at 6.4×10^{-9} ampere full scale dimension (afs) in the FID.

Spectrometry

The infrared (IR), ultra-violet (UV) and proton nuclear magnetic resonance (PMR) spectra were recored on Beckman model IR-20A, Hitachi model EPS-3T and Varian FT80A (80MHz) spectrometer, respectively. The MTM-barbiturates were examined neat to obtained the IR spectra and the PMR spectra were reported in part per million (ppm), relative to internal standard, tetramethylsilane. The mass spectra were measured on Hewlett-Packard model 5985B spectrometer coupled to Hewlett-Packard model HP5840A gas chromatograph operating on an OV -101 fused silica capillary column (12m×0.2mm i.d.) and were obtained with an ionizing voltage of 70eV, an electron-multiplier valtage of 1.8KV, a carrier gas (helium) linear flow rate of 20cm/sec, an ion source temperature of 200℃ and the column temperature of 210℃.

Standard Preparation

Accurately weighed about 100 μmol of barbiturate standards (barbital, allobarbital, phenobarbital, secobarbital and thiopental), transferred to a 25-ml volumetric flask and diluted to the volume with ethanol, pipetted 0, 6, 12, 18, 24 and 30 μl of the solution into reaction vials with aid of a 50-μl Hamilton microsyringe, dried under stream of nitrogen at 70℃ and added 1ml of dry HMPA and 1ml of sodium hydride. After stirring for 30 min, mixed with 100 μl of methylthiomethyl chloride and then shaked for 24 hours. The mixture was partitioned between 3ml of benzene and 3ml of water. After removal of the aqueous layer, the organic phase was dried on anhydrous sodium sulfate and the supernatant was taken for GC analysis.

For investigation of the effect of sulfur-non-containing interference on analysis of barbital, the final supernatants spiked with HAME at 0, 1, 10 and 100 nml/ml were used and then solutions were evaporated to dryness with aid of nitrogen current. The residues were dissolved in 1ml of ethanol and then injected on 3% OV-1 column. Determinations were calculated by a corrected response equation (17) in case of flame photometric detection and by a half-height width method in case of flame ionization detection.

Plasma Assay

Plasma was seperated from rat whole blood by centrifugation. Plasma samples spiked with barbiturates at 0, 40, 80, 120, 160 and 200 nmol/ml were used. To the spiked specimens in each 15-ml centrifuge tube were added 1ml of 1N -HCL and 5ml of isopropyl ether-benzene mixture (2 : 1). These mixtures were vortexed for 15 seconds and then centrifuged at 3000 rpm for 5min. The orgainc phases were transferred to another 10-ml screw-capped test tubes and stood on a little amount of anhydrous sulfate for a short time. Pipetted 3ml of the supernatants into reaction vials and then proceeded as under "Standard Preparation" beginning with "dried under a stream of nitrogen at 70℃ ---" and ending with "---the supernatant was taken for GC analysis".

Isolation of pure MTM-barbiturate

The preparative thin-layer chromatography (TLC) of the resulting products was carried out on the plate coated with Kieselgel 60HF254 (0.75 mm thick layer) for collection of pure MTM-barbiturates, and the analytical TLC was carried out on the precoated plate with Kieselgel 60F254. The developping solvent used was a mixture of hexane and ethylacetate (7 : 3) and visualization was performed with short wave UV light. After no impurities in the collected substances were confirmed by the GC-FID method and the substances were dried by a freeze drier (Edwards moded EF03, British Oxygen Co., Ltd.), those substances were identified by PMR, IR and mass spectra.

Rf values of MTM- barbiturates in conditions above and UV (absorbance maxima) (in parenthesis) were as following: MTM-barbital: 0.77 (232 nm); MTM-allobarbital: 0.83 (230nm); MTM -phenobarbital: 0.75 (234nm, 2.45nm); MTM -secobarbital: 0.86 (236nm); MTM-thiopental: 0.90 (248nm, 289nm).

RESULTS AND DISCUSSION

Synthesis and Identification of MTM-Barbiturate

Amide or imide is very base, far too weak to attack alkyl halides, so that it must first be converted to its salts. Therefore only malonylurea salt

reacts with methylthiomethyl chloride to give MTM-barbiturate.

The MTM-barbiturate collected by the preparative TCL was yellow viscous oil. Proof of N,N' -dimethylthiomethyl formation rather than 2-methylthiomethyl ether formation was obtained by the use of the PMR, IR and mass fragmentography. As shown in Fig.1, the PMR spectra of MTM -barbital, -allobarbital, -secobarbital and -phenobarbital showed singlet resonances at 4.94, 4.92, 4.91 and 1.95 ppm, respectively, and that of MTM -thiopental showed a singlet resonance at a lower field, 5.44 ppm. It is considered that they were attributed to either $=NCH_2S$ protons or XCH_2S (X=O or S) protons. It is noted that the alkyl protons attached to ether group resonance at a lower field than those attached to thioether group and that the N-alkyl protons bonded to ureide resonance at an upper field than those bonded to thioureide in the PMR spectroscopy. Accordingly the PMR spectra

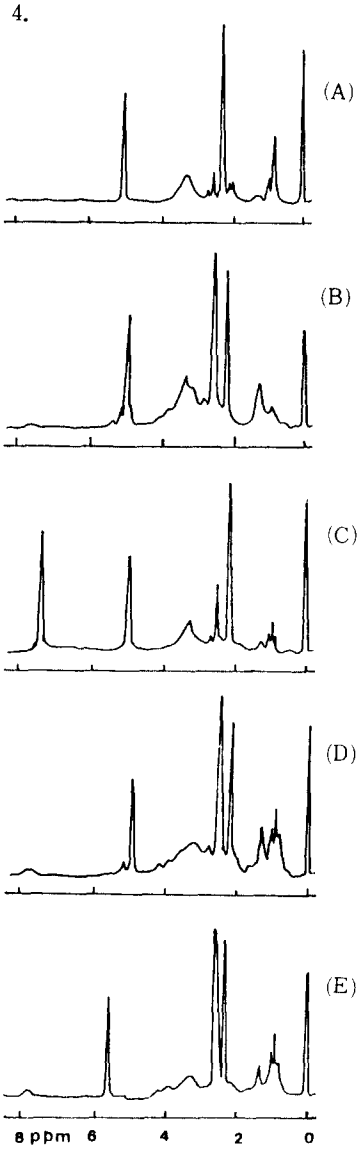

Fig.1. PMR spectra of MTM derivatives of barbital(A), allobarbital(B), phenobarbital(C), secobarbital(D) and thiopental(E).

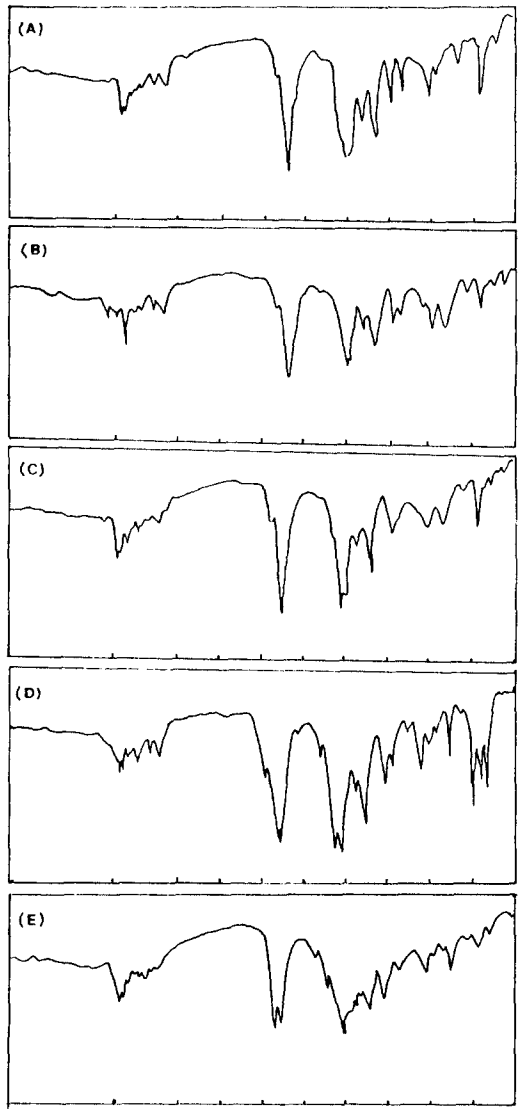

Fig.2. IR spectra of MTM derivatives of barbital(A), allobarbital(B), secobarbital(C), phenobarbital(D) and thiopental(E).

of MTM-barbiturate in DMSO-d6 solution clearly indicated N-methylthiomethyl derivative formation. The peaks due to the imide proton did not appear in the PMR spectra. That is, this fact suggests that the resulting product is N,N'-dimethylthiomethyl barbiturate.

In the IR spectra (Fig.2), the strong bands were not present at 3250-3300cm^{-1} assigned to the NH streching vibrations. So good supporting evidence was adduced in favor of N,N'-dimethylthiomethyl derivative rather than N-methylthiomethyl or methylthiomethyl ether derivative.

The mass fragmentography was carried out as further proof of derivative formation. As shown in Fig.3 and Table I, the mass data exhibit base peak at m/z 61 due to $CH_2=S-CH_3$ ion, the characteristic peak at m/z 102 due to $CH_2=S-CH_3-N=C=O$ ion and the peak at m/z 118 due to $CH_2=S-CH_2-N=C=S$ ion in MTM-thiopental. The formation of these ions suggests that the result-

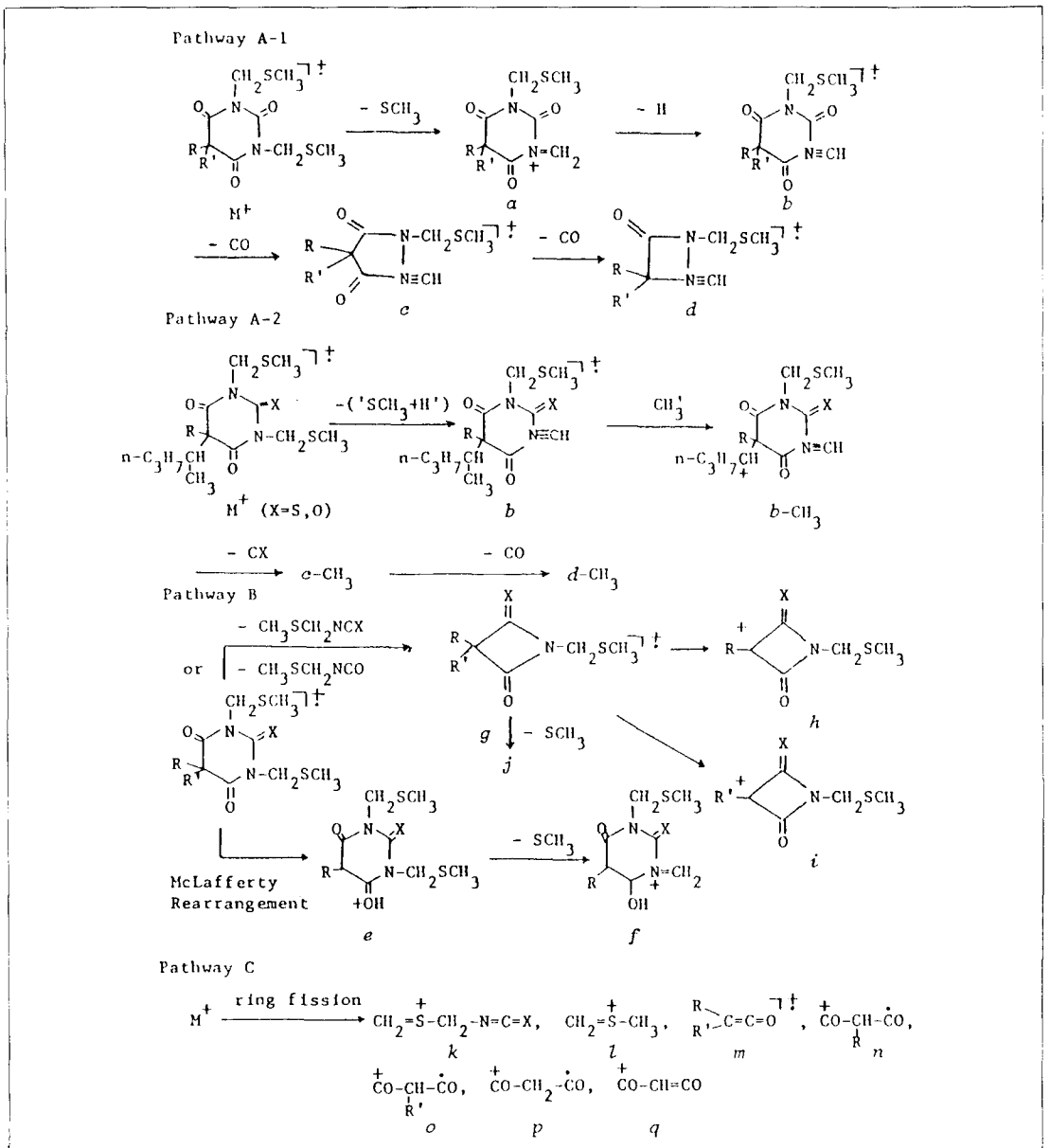

Fig.3. Fragmentation pathway of MTM-barbiturates.

Table I. Mass fragment ions of barbiturates.

Compound	Fragment Ions, m/z (% relative abundance)										
	M^+	M^+-CH_3	*a*	*b*	*b*-CH_3	*c*	*c*-CH_3	*d*	*d*-CH_3	*e*	*f*
Barbital	304 (1.8)		257 (4.4)	256 (19.5)		228 (0.8)		200 (2.4)		276 (0.3)	229 (0.2)
Allobarbital	328 (1.9)		281 (0.7)	280 (0.5)		252 (0.2)		224 (0.2)		288 (0.1)	241 (0.4)
Phenobarbital	352 (0.2)		305 (0.7)	304 (3.8)		276 (0.2)		248 (0.2)		276 (0.2)	229 (0.8)
Secobarbital	358 (0.0)	343 (0.2)	311 (0.0)	310 (0.0)	295 (0.1)	282 (0.0)	267 (6.6)	254 (0.0)	239 (1.4)	288 (0.3)	241 (0.4)
Thiopental	362 (0.0)	347 (0.9)	315 (0.0)	314 (0.0)	299 (9.1)	270 (0.0)	255 (0.4)	242 (0.0)	227 (0.1)	292 (0.1)	245 (0.2)

Compound	Fragment Ions, m/z (% relative abundance)										
	g	*h*	*i*	*j*	*k*	*l*	*m*	*n*	*o*	*p*	*q*
Barbital	201 (0.3)	172 (0.3)	172 (0.3)	154 (3.5)	102 (15.7)	61 (100)	98 (8.2)	98 (8.2)	98 (8.2)	70 (10.8)	69 (11.2)
Allobarbital	225 (0.2)	184 (0.5)	184 (0.5)	178 (0.6)	102 (9.6)	61 (100)	122 (1.2)	110 (0.5)	110 (0.5)	70 (4.4)	69 (0.5)
Phenobarbital	249 (0.0)	172 (0.4)	220 (0.4)	202 (0.5)	102 (11.8)	61 (100)	146 (22.0)	98 (0.2)	146 (22.0)	70 (4.8)	69 (1.0)
Secobarbital	255 (0.2)	184 (0.7)	214 (0.2)	208 (0.5)	102 (9.2)	61 (100)	152 (2.8)	110 (2.2)	140 (0.4)	70 (4.0)	69 (4.0)
Thiopental (X=O)	243 (0.1)	172 (0.4)	214 (0.2)	196 (0.3)	102 (3.8)	61 (100)	140 (0.4)	98 (1.1)	140 (0.4)	70 (0.9)	69 (7.9)
X=S)	259 (0.0)	186 (0.2)	230 (0.0)	212 (0.2)	118 (3.3)						

ing products were not an methylthiomethyl ether derivatives but an N-methylthiomethyl derivatives. Consequently, it is concluded from the PMR, IR, and mass fragmentography that both the N-1 and N-3 position of hydropyrimidine ring were methylthiomethylated.

The molecular ions at m/z 304 (MTM-phenobarbital), or the ions at m/z 343 (MTM-secobarbital), and at m/z 347 (MTM-thiopental) attributed to the loss of methyl radical from isopropyl group in molecular ion are in agreement with the confirmed structures. Formation of the mass ions is postulated in Fig.3. The proposed fragmentation pathway A is similiar to those of N-methylated catechol metabolites of phenytoin suggested by Midha *et al.* (18) and the rationale of pathway B is consistent with the earliar literature (19). The fragmentation pathway C is explained in terms of simple ring fissions as shown in the literature (20).

Analytical Studies and Yield

When about 10 equivalence of methylthiomethyl chloride was used versus sodium barbiturate suspended in HMPA, MTM-barbiturate was almost quatitatively yielded as shown in Table I.

MTM-thiopental and MTM-phenobarbital were overlapped on 3% OV-1 column but all MTM barbiturates were exellently separated on 3% OV-225 column within 16 min (Fig.5).

Fig.4 shows standard calibration curves of barbiturates by the GC-FPD method. The relationship between the concentration of barbiturate and the corrected response value for peak area was given by barbital; $Y=4.335X+0.016$ ($r=0.9984$), allobarbital; $Y=4.258X+0.120$ ($r=0.9989$), secobarbital; $Y=4.250X+0.021$ ($r=0.9997$), phenobarbutal; $Y=4.329X-0.091$ ($r=0.9985$) and thiopental; $Y=6.431X-0.017$ ($r=0.9992$) where X is the concentration and Y is the corrected response value. The calibration curves were satisfactorily linear within the concentration range from 10 nmol/ml to 40 nmol/ml. The slopes and Y-intercepts of four calibration curves except that of MTM-thiopental did not differ significantly ($F\alpha(4, 16; m)=1.79$), as determined by F-test (21). Sample data of four MTM-barbiturates with two sulfur-atoms were

Table II. Formation yields of MTM-barbiturate.

Compound	1st Run(mg)		2nd Run(mg)		3rd Run(mg)		Yield (%)
	Added	Found	Added	Found	Added	Found	
Barbital	21.2	20.1	20.3	20.8	19.9	19.0	97.6
Allobarbital	20.7	18.2	19.8	20.2	21.4	20.6	94.0
Secobarbital	24.2	24.3	25.5	23.4	26.0	24.2	95.1
Phenobarbital	27.6	16.4	25.0	25.5	26.3	24.4	96.8
Thiopental	13.8	13.6	13.6	12.7	12.9	12.7	94.7

*Estimated by GC-FPD.

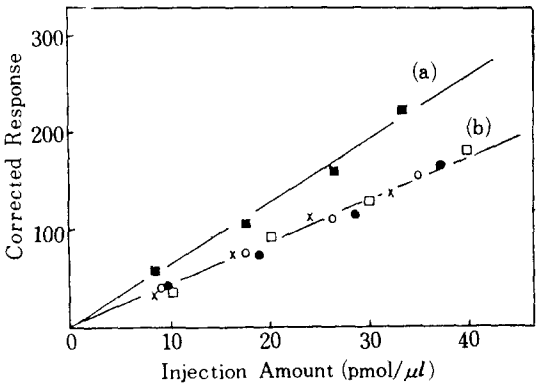

Fig.4. Calibration curves of thiopental(■—■), barbital(×—×), allobarbital(□—□), secobarbital(○—○) and phenobarbital (●—●) obtained by GC-FPD.

fitted to reduced model by the method of least squares (21) and then the fitted calibration curves is obtained as shown in Fig.4. The XY relationship was Y=4.284X+ 0.062 (r=0.9987).

The ratio of slope of MTM-thiopental calibration curve to that of slope of MTM-thiopental calibration curve to that of the fitted calibration curve was 1.502 and almost agreed with the ratio (3/2=1.5) of sulfur-atom number of MTM -thiopental to that of other MTM-barbiturate.

Plsma Assay

As shown in Fig.5, no interferences were detected in control plasma by the FPD. Extraction recovery of barbiturate from rat plasma was measured by the GC-FPD method and GC-FID method and listed in Table III. Differences between recoveries by both detection methods were not statistically significant ($F(2,8;\alpha)<0.72$).

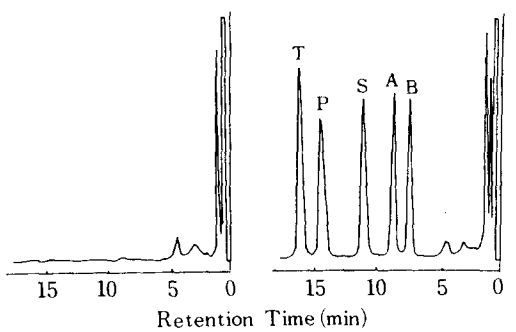

Fig.5. Gas chromatograms of rat Plasma by GC -FPD. Left-Control plasma; Right -Plasma spiked with 24nmol/ml of barbital(B), allocarbital(A), secobarbital(S), Phenobarbital(P) and thiopental(T).

Table III. Extraction recovery of barbirurates from rat plasma.

Compound	Method	*Recovery(%)	r
Barbital	FPD	99.0±5.6	0.9992
	FID	100.4±4.9	0.9994
Allobarbital	FPD	94.5±8.0	0.9981
	FID	95.9±5.6	0.9991
Secobarbital	FPD	92.5±5.2	0.9992
	FID	91.4±1.6	0.9999
Phenobarbital	FPD	80.5±5.0	0.9989
	FID	83.8±5.6	0.9986
Thiopental	FPD	83.1±8.3	0.9974
	FID	82.0±4.5	0.9992

*Percentage of slope calculated by the method of least squares (n=6, 0~200 nmol/ml).

The correlation coefficients (r) obtained by the GC-FPD method indicated tighter fit of the data to the regression lines than those by the GC-FID method as shown in Table 3. But it should be noted that data were obtained with the amplifier sensitivity range setting at 6.4×10^{-9} afs in the FPD and 1.6×10^{-9} afs in the FID. This indicates that, for MTM-barbiturates, the FPD is at least four times more sensitive than the FID. The minimum detectable amount of MTM-barbiturate with two sulfur-atoms on this column was about 10.0

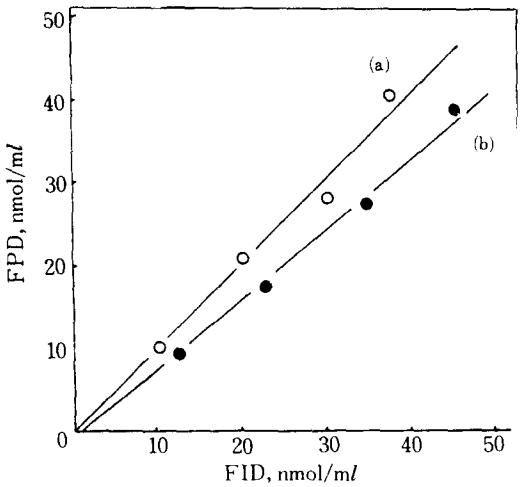

Fig.6. Comparison of standard barbital levels by GC-FPD and by GC-FID. Curve(a) was resulted from MTM-barbital only, and curve(b) from the mixture of MTM-barbital and HAME(10nmol/ml).

fmol/injection. Where sulfur compound and sulfur -noncontaining interference were overlapped on GC column, the analytical properties afforded by two detection methods were investigated. MTM -barbital sample spiked with HAME were used and determinded on 3% OV-1 column. HAME was not detected by the FPD. On FID-gas chromatogram, the area of MTM-barbital peak was calculated by subtracting the area of HAME peak from total peak area. Fig.6 shows correlations between the concentrations of MTM-barbital measured by these methods. Line (a) is resulted from MTM -barbital only and gives a slope of 0.02 (r=0.9273) and an intercept of 0.07 nmol/ml, line (b) is resulted from the mixture of MTM-barbital and HAME and gives a slope of 1.13 (r=0.9096) and an intercept of −2.26 nmol/ml. These data suggest that the differences of the slopes and intercepts may be due to overestimation by the FID method.

Table IV shows an effect of sulfur-noncontaining interference, HAME, on recovery for MTM -barbital by the GC-FPD method.

These data indicate that the concentration of MTM-barbital measured by the GC-FPD method is underestimated if over 5 equivalence of HAME is mixed versus MTM-barbital. This cause hydrocarbon backgroud in the flame (22, 23). The sulfur selectivity (24) to hydrocarbons is 10^4-10^5 and so determination of MTM-barbiturate could be carried out by this method unless a fixed excess of sulfur-noncontaining compound was mixed even though two compound was mixed even though two compounds are overlapped on GC column.

Table IV. Effect of heptadecanoic acid methyl ester (HAME) on recovery for MTM -barbital by GC-FPD.

HAME (nmol/ml)	MTM-barbital (nmol/ml)		Recovery (%)
	Added	Measured	
0	7.84	8.12	103.6
	23.52	22.79	96.9
	39.20	39.80	101.5
1	7.84	7.75	98.9
	23.52	23.60	100.4
	39.20	38.96	99.4
10	7.84	7.47	95.3
	23.52	23.20	98.6
	39.20	38.42	98.0
100	7.84	6.67	85.1
	23.52	21.58	92.2
	39.20	37.93	96.8

CONCLUSION

The barbiturates in plsma were quantitatively N, N'-dimethylthiomethyl-derivatizaed and could be determined at a minimum detectable amount of the GC-FPD method. Using one barbiturate standard calibration curve, it was possible to determine other barbiturates by this method. The results suggest that the FPD also produced chromatogram with less background than that obtained with the FID for extracted plasma sample and that the sulfur compound could be assayed by this method unless over ca. 5 equivalence of hydrocarbon impurities was overlapped on GC column.

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