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Enzymic Digestion of Biosamples as a Method of Sample Pretreatment Before XAD-2 Extraction*

M. Bogusz, J. Bialka, and J. Gierz

Institute of Forensic Medicine, Academy of Medicine, PL-31531 Krakow, Grzegorzecka 16, Poland

Summary. Four proteolytic enzymes: trypsin, protease type I, protease type V (Pronase AS) and protease type VIII (Subtilisin) were used for digestion of blood, brain, liver, and kidney in experimental poisonings of dogs with phenobarbital, amitriptyline, diazepam, and chlorpromazine. Enzymic digestion was used also in 21 forensic cases of fatal poisonings with hypnotic and psychotropic drugs. Each enzyme applied gave a similar yield. The efficiency of enzymic digestion varied according to the kind of drug, tissue, and individual. In general, enzymic hydrolysis proved useful in isolation of psychotropic drugs from liver and brain, being less effective in the case of blood. The mechanism of drugs liberation after enzymic treatment remains obscure.

Key words: XAD-2 extraction – Enzymic digestion – Psychotropic drugs, enzymic digestion

Zusammenfassung. Vier proteolytische Enzyme: Trypsin, Protease Typ I, Protease Typ V (Pronase AS) und Protease Typ VIII (Subtilisin) wurden für die Spaltung von Blut, Gehirn-, Leber- und Nierengewebe verwendet, nachdem man Hunde experimentell mit Phenobarbital, Amitriptylin, Diazepam und Chlorpromazin vergiftet hatte. Die enzymatische Spaltung wurde weiterhin bei 21 forensischen Fällen mit tödlicher Vergiftung durch Schlafmittel und psychotrope Drogen angewandt. Jedes verwendete Enzym erbrachte ein ähnliches Resultat. Die Effizienz der enzymatischen Spaltung variierte entsprechend der Art der Droge, Gewebe und Individuum. Die enzymatische Hydrolyse zeigte sich nützlich bei der Isolation psychotroper Drogen aus Leber und Gehirn, sie war weniger effektiv im Falle von Blut. Der Mechanismus der Drogenfreisetzung nach enzymatischer Behandlung bleibt ungewiß.

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Offprint requests to: Dr. M. Bogusz (address see above)

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Schlüsselwörter: XAD-2-Extraktion – enzymatische Spaltung – psychotrope Drogen, enzymatische Spaltung

Drugs which entered the body are extensively bound to the plasma and tissue proteins. The character of binding may vary according to the kind of drug and kind of tissue. Plasma proteins, mainly albumins, link drugs by non-covalent bonds. The affinity and strength of the linkage vary from weak to extremely strong, which may be disrupted only by destroying the ligand or protein molecule. It should be added that drugs are often bound more avidly to proteins in tissues than in plasma [1].

These problems are well known to forensic toxicologists and create many problems concerning the isolation of drugs from tissues. A routine approach, which enables liberation of drugs from tissues, is acid hydrolysis. Enzymic hydrolysis with proteolytic enzymes appeared to be an interesting alternative to this procedure, assuring more mild conditions of sample pretreatment [2–5]. The method appeared most effective for liberation of drugs from organs, particularly the liver.

The purpose of this paper was to present our own experience on enzymic digestion of biosamples in experimental and authentic drug poisonings. The extraction of drugs with Amberlite XAD-2 resin, applied in the study, was proven by various authors to be a convenient and efficient method of isolation [6–11], helping to find a broad spectrum of drugs.

Material and Methods

The experimental part of study was performed on four mongrel dogs which were given orally: 200 mg phenobarbital/kg b.w., 100 mg amitriptyline/kg b.w., 100 mg chlorpromazine/kg b.w., and 150 mg diazepam/kg b.w. After 24 h the animals were killed, the samples of blood, liver, brain, and kidney were taken and stored at −20°C. From this stock samples were taken several times (at least twice) for analysis.

The homogenate of biologic material was either deproteinized by heating in a steam bath without any pretreatment in the case of phenobarbital poisoning, or hydrolyzed with 5 n HCl in

Table 1. Properties of proteolytic enzymes used for digestion of blood and tissues

Enzyme	Protease I	Protease V	Protease VIII	Trypsin
Source	Pancreas	Strept. gris.	Bacc. subtilis	Pancreas
Proteolytic activity	Trypsin- and chymo-trypsin-like activity		Leu-Tyr, Glu-His, Ser-His, Leu-Val	Arg–, Lys–
Optimum pH	7–9	7–9	10–11	7–9
Specific activity	9 U/mg	1.1 U/mg	11 U/mg	35 U/mg
Preparation	Lyophilized powder			
Cost per analysis (US c)	5	55	20	5

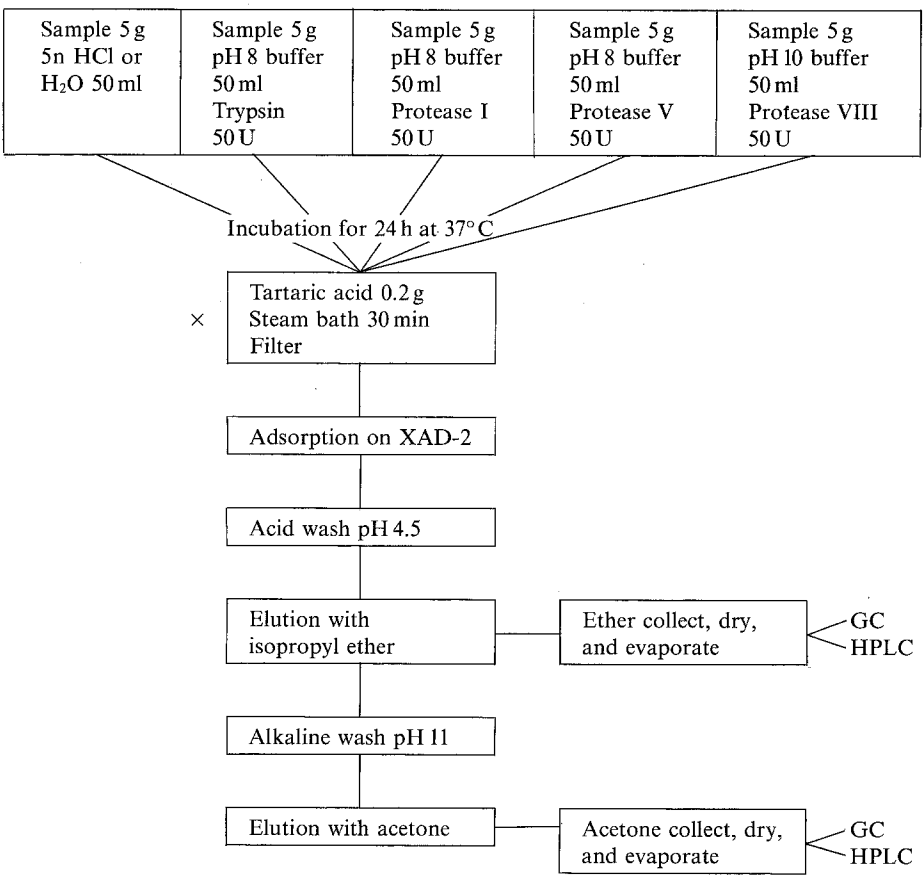

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Fig. 1. Flow diagram of applied methods of drug isolation

other poisonings, or digested with several proteolytic enzymes. The properties of these enzymes are shown in Table 1. The enzymes were supplied by Sigma Chem. Co. (USA). Five-gram portions of samples were digested with 10 U of each enzyme per 1 g of sample for 24 h at 37° C. The control samples were incubated in the same conditions without enzyme. After incubation samples were deproteinized and adsorbed on XAD-2 resin and eluted with isopropyl ether and acetone, using XAD-2-Bag technique described elsewhere [12]. Dry residues of eluates were examined by means of gas chromatography and high-pressure liquid chromatography. Gas chromatographic determinations were performed on Pye GCD chromatograph equipped with 3% OV-17 and 3% SE-30 columns. For HPLC examinations KB-1101 chromatograph was applied with columns filled with ODS-C_{18} phase (Fig. 1).

Apart from experimental material, the investigations were carried out in 21 cases of fatal poisonings with hypnotic and psychotropic drugs. In these cases the same enzymes were used for digestion, and the drugs were isolated by XAD-2-Bag technique [12] or by XAD-2 micromethod [13]. Also the blank samples taken from non-poisoned subjects were analyzed, serving as a source of data concerning the biologic background of various methods of isolation and detection. Drugs were detected and quantitated by means of gas chromatography with flame ionization (Pye GCD) or nitrogen-sensitive detector (Perkin-Elmer F 22) and by high pressure liquid chromatography.

	Blood	Brain	Kidney	Liver
Control	1	1	1	1
Trypsin	0.9	1.3	0.6	3.0
Protease I	1.2	2.3	0.6	2.1
Protease V	1.1	3.1	0.4	3.6
Protease VIII	0.8	2.8	0.4	4.0

Table 2. Phenobarbital concentrations in blood and tissues treated by various methods. Relative values: concentration of drug in non-digested sample = 1

	Blood	Brain	Kidney	Liver
Control	1	1	1	1
Trypsin	0.9	1.6	1.4	3.4
Protease I	0.6	1.1	1.1	3.4
Protease V	0.4	2.2	1.1	5.2
Protease VIII	1.5	2.7	1.2	6.3

Table 3. Amitriptyline concentrations in blood and tissues treated by various methods. Relative values: concentration of drug in non-digested sample = 1

Table 4. Chlorpromazine (CPZ) and 7-OH-chlorpromazine (OH-CPZ) in blood and tissues treated by various methods. Relative values: concentration of drug in non-digested sample = 1

	Blood		Brain		Kidney		Liver	
	CPZ	OH-CPZ	CPZ	OH-CPZ	CPZ	OH-CPZ	CPZ	OH-CPZ
Control	1	1	1	1	1	1	1	1
Trypsin	1.0	3.6	3.0	2.1	1.6	1.3	3.9	0.7
Protease I	1.1	2.9	2.4	2.4	2.3	1.0	1.8	1.2
Protease V	1.0	2.7	2.2	1.5	2.0	1.3	1.4	1.5
Protease VIII	1.4	3.0	1.7	1.7	3.9	2.7	1.8	0.8

In each case, TLC- and UV-spectrophotometric examination was also performed. In the case of morphine intoxication radioimmunoassay (Abuscreen-Roche) was applied. The diphenhydramine metabolite was determined by means of GC/MS using Hewlett-Packard 5990 gas chromatograph-mass spectrometer equipped with 3% Dexsil column, and operated on the selected ion monitoring mode. The concentration of metabolite was calculated from m/e ions 167.00 and 183.00 [14, 15].

Results

The examination of material from experimental phenobarbital poisoning shows that the enzymic digestion of brain and liver allowed to detect distinctly greater amounts of drug (Table 2). On the other hand, the concentrations of phenobarbital

Table 5. Diazepam (D) and N-desmethyldiazepam (ND) concentrations in blood and tissues treated by various methods. Relative values: concentration of drug in non-digested sample = 1

	Blood		Brain		Kidney		Liver	
	D	ND	D	ND	D	ND	D	ND
Control	1	1	1	1	1	1	1	1
Trypsin	0.5	0.3	1.8	0.6	1.4	0.3	1.7	6.9
Protease I	1.0	0.8	3.0	2.3	1.0	0.1	1.0	1.1
Protease V	0.5	0.4	4.0	1.8	1.1	0.5	1.1	2.5
Protease VIII	1.0	0.8	1.9	3.0	1.1	0.6	0.7	2.3

Table 6. Concentrations of hypnotic drugs found in fatal cases after various methods of sample pretreatment (mg/l)

Drug	Tissue	Control	Trypsin	Protease I	Protease V	Protease VIII
Phenobarbital	Blood	80	120			
	Liver	63	110			
	Brain	53	67			
Cyclobarbital	Blood	3	18	13	16	22
	Liver	14	21	23	17	12
	Brain	2	7	8	7	3
Gluthetimide	Blood	3	1	1	1	0.5
	Liver	5	7	3	3	10
	Brain	4	1	2	4	3
	Kidney	4	7	4	6	3
Methaqualone	Blood	11		18		
	Liver	50		282		
	Blood	2		3		
	Liver	9		10		
	Brain	3		3		

in the blood were similar, irrespective of the method of pretreatment, and in the kidneys the results obtained after enzymic digestion were even worse than in the controls. Enzymic digestion of liver and brain was also effective in experimental amitriptyline poisoning (Table 3). The values obtained from blood and kidney did not differ significantly from the controls. Distinctly greater amounts of chlorpromazine were found after enzymic hydrolysis of brain, kidney, and liver (Table 4).

This procedure was also effective for isolation of metabolite-7-OH-chlorpromazine from blood and brain. The examination of tissues taken from dog poisoned with diazepam show that enzymic digestion was effective only in brain

Table 7. Concentrations of phenothiazines found in fatal cases after various methods of sample pretreatment (mg/l)

Drug	Tissue	Control	Trypsin	Protease I	Protease V	Protease VIII
Chlor-promazine	Blood	1.4		4.6	2.1	1.8
	Liver	1.3		4.6	2.5	3.4
	Brain	1.4		8.0	4.5	6.1
Promethazine	Blood	3.6	2.7			
	Liver	31.1	140.6			
	Brain	6.6	5.6			
Methotrime-prazine	Blood	0	22.0			
	Liver	8.2	56.0			
	Brain	6.0	16.9			

Table 8. Concentrations of morphine, amphetamine and diphenhydramine in samples digested with enzyme and in controls

Drug	Tissue	Control	Protease I
Morphine (ng/ml)	Blood	440	500
	C.s. fluid	660	640
	Liver	380	470
	Brain	230	160
Amphetamine (mg/l)	Blood	1.1	1.9
	Liver	1.2	4.1
	Brain	1.9	1.8
Diphenhydramine (mg/l)	Blood	0.4	1.7
	Blood	4.6	31.9
	Blood	5.0	23.8
	Liver	48.2	48.8
Diphenhydramine metabolite (mg/l)	Blood	6.6	5.3
	Blood	3.1	3.4

(Table 5). In other organs and in blood the quantities of drug found by various methods were similar. The yield of N-desmethyldiazepam was higher after enzymic treatment of liver and brain. Table 6 illustrates the results of toxicologic investigations in fatal cases of poisonings with some hypnotic drugs. Higher values were obtained after enzymic treatment of tissues in barbiturate poisonings, whereas this methods was not effective in gluthetimide intoxication. In one case of methaqualone poisoning enzymic digestion was successful, whereas in the second case it was not effective. Proteolytic enzymes were effective in the examination of material taken from subjects poisoned with phenothiazines (Table 7). The results of

Table 9. Comparison of results obtained in cases of fatal drug poisonings. Better results were obtained if the concentration found after enzymic digestion was at least 1.5-fold higher than in control samples

Better result in enzymic digestion	Better or the same result in controls
Phenobarbital	Gluthetimide
Phenobarbital	Carbamazepine
Phenobarbital	Chlorprothixene
Phenobarbital + gluthetimide	Imipramine + diazepam
Cyclobarbital	Methaqualone
Cyclobarbital + chloropromazine + oxazepam	Morphine
Cyclobarbital + amidopyrine + oxazepam	Morphine
Methaqualone	
Diphenhydramine	
Diphenhydramine	
Diphenhydramine	
Methotrimeprazine	
Methitrimeprazine + amitriptyline	
Amphetamine	

investigations in amphetamine, morphine, and diphenhydramine poisonings are shown in Table 8. Digestion proved useful in the case of blood samples in diphenhydramine poisoning. In morphine intoxication the enzymic hydrolysis did not improve the yield. It should be stated additionally that proteolytic enzymes did not affect the antigen-antibody reaction applied for morphine determination.

Table 9 shows the sum of results obtained by the examination of 21 cases of fatal poisonings with various drugs. In 14 cases digestion with proteolytic enzymes enhanced the yield of drugs from tissues.

Blank samples treated with proteolytic enzymes showed a considerable background in UV and GC/FID examination. UV-spectrophotometry of liver samples digested with enzymes revealed phenolic-like spectra which are usually observed in putrefied tissues. On the other hand, the analytic background in GC/NPD and HPLC examinations was negligible and did not affect the interpretation of chromatograms.

Discussion

Investigations carried out on experimental material and in forensic cases have shown that enzymic digestion of liver and brain may cause a higher yield of some psychotropic and hypnotic drugs. In the case of blood, enzymic digestion was not effective, only in diphenhydramine poisoning.

Each enzyme used: trypsin, pancreatic protease type I (which in fact is a mixture of trypsin and chymotrypsin), bacterial protease type V and Subtilisin, gave a similar yield in examined material. Comparatively low cost of trypsin and protease type I suggests that one of these enzymes be chosen for routine application.

It should be stressed that the efficiency of enzymic hydrolysis varied according to the kind of drug and tissue; even individual variability was observed. This confirms our previous observations concerning enzymic digestion with trypsin and beta-glucuronidase [5], when this procedure was highly effective in some intoxications and without any effect in others. Dickson [16] observed variability in elution efficiency according to the kind of eluting solvent applied after Subtilisin digestion.

Kauert et al. [17] have shown that digestion with Subtilisin had no influence on the liberation of protein-bound nordiazepam from spiked autopsy blood. Meyer et al. [18] found that Subtilisin treatment improved the yield of drugs from blood extracted with XAD-2 resin, but was not effective before extraction with Extrelut columns. Also Machata (pers. commun.) stated that enzymic digestion caused no improvement when applied before ultrasonic extraction. Kauert et al. [17] presumed that Subtilisin digestion has no influence on drug-protein linkage, but only improves the liquefaction of samples. In view of the present study, this hypothesis seems probable. It is possible that enzymic digestion causes mild and exact homogenization of biologic material, particularly organs, and facilitates the contact between drug and extrahent. This assumption may be supported by the observation that the yield of drugs isolated from the blood did not increase after enzymic hydrolysis, and that the enzymic digestion was not effective in the isolation of drugs particularly strongly bound, such as morphine, imipramine, or diphenhydramine metabolite.

In final conclusion it may be stated that despite lack of knowledge of action, enzymic digestion should be applied in forensic toxicology because of simplicity, low cost, and potential effectiveness.

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