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The extraction and analysis of benzodiazepines in tissues by enzymic digestion and high-performance liquid chromatography

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A simple, rapid and sensitive method is described for the analysis of unchanged benzodiazepine drugs and their metabolites, in human and animal tissues, using the proteolytic enzyme subtilisin Carlsberg followed by reverse-phase liquid chromatography. The enzymic digestion yields far higher recoveries than conventional extraction methods, while the liquid chromatographic analysis allows the rapid separation and detection of nanogram quantities of these drugs without the need for elaborate "clean-up" procedures.

Analysis of benzodiazepines in tissues presents difficulties since these compounds are bound tightly to body proteins (Kaplan, Jack & others, 1973; Zingales, 1973) and are also acid labile. Usual methods of analysis involve acid hydrolysis of tissues with conversion of benzodiazepines into benzophenones (Clifford & Franklin Smythe, 1974) but many benzodiazepines are converted into the same benzophenone (Jackson, 1969; Hailey, 1974).

This paper describes the use of the non-group specific proteolytic enzyme subtilisin Carlsberg to degrade animal and human proteins thus releasing unchanged benzodiazepines into solution from which they may be extracted. Analysis of such drugs in tissue extracts may then be achieved using reverse-phase liquid chromatography without lengthy 'clean-up' procedures. The procedure has been successfully applied to chlordiazepoxide, diazepam, flurazepam, lorazepam, medazepam, nitrazepam, oxazepam, potassium clorazepate and their metabolites.

MATERIALS AND METHODS

Enzymic digestion of liver tissue by subtilisin Carlsberg

Subtilisin Carlsberg (Crystalline bacterial alkaline protease EC 3.4.4.14) manufactured by Nagase & Co Ltd. Japan, was obtained from the Digby Chemical Service, London. Liver aliquots (1 g and 10 g) were blended in 1·0 M, pH 10·5 tris base (15 and 25 ml respectively) and then incubated with subtilisin (1·0 mg subtilisin g^{-1} liver) at 50°–60° (60 min) with continuous magnetic stirring. The resulting solutions were cooled to room temperature (20°), filtered through a small plug of loosely packed glass wool to remove any undigested blood vessels or connective tissue, and the filtrates extracted by vigorous shaking with 2 × 200 ml diethyl ether. The extract was dried (Na_2SO_4), the ether evaporated and the residue dissolved in absolute ethanol (2 ml ethanol for 1–5 g liver samples and 4 ml ethanol for 10 g liver samples). Microlitre volumes were analysed for benzodiazepines using reverse-phase liquid chromatography. Blank extracts were prepared from drug-free guinea-pig or human livers.

Analysis of benzodiazepines in liver by conversion to their respective benzophenones

Liver tissue (10 g) was homogenized with water (20 ml) before the addition of conc HCl (20 ml). The mixture was heated by immersion in boiling water for 90 min then filtered using loosely packed glass wool. The cooled digest was extracted with diethyl ether (2 × 150 ml) and the combined ether extracts washed three times with 25 ml NaOH (2 M). The organic layer was dried (Na_2SO_4), the ether evaporated and the residue dissolved in 1·0 ml H_2SO_4 (1·0 M) before analysis by ultraviolet spectrophotometry and h.p.l.c.

High-performance liquid chromatography (h.p.l.c.)

A constant flow pump (M-600, Waters Associates, Stockport, Great Britain) was used to deliver eluent to a column (150 × 4·6 mm i.d.) slurry-packed with Spherisorb-5-ODS (Phase Separations, Queensferry, Clwyd). Samples for analysis were injected by μl syringe onto a layer of glass beads at the head of the analytical column. A modified ball valve (Type 7115G4Y, Hoke International, New Barnet, Herts) was used as a stop-flow injector. Eluents were prepared from aqueous 0·025 M disodium hydrogen phosphate and methanol (AR) and adjusted to pH 7·8 with H_3PO_4 (10% w/v). In general, eluents containing 60% methanol were used at a flow rate of

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1·0 ml min^{-1}. For medazepam, 70% methanol (1·0 ml min^{-1}) was used, and for flurazepam the analysis time was reduced by using 80% methanol at a flow rate of 2·0 ml min^{-1}. A flow of 1 ml min^{-1} generated a back-pressure of 6·9 MPa (1000 lb in^{-2}).

Benzodiazepines and their metabolites were detected by monitoring the column eluent at 254 nm with a variable-wavelength ultraviolet monitor (CE-212, Cecil Instruments, Cambridge); those fractions corresponding to the benzodiazepine peaks were collected and their identity confirmed by their ultraviolet absorption spectra and g.l.c. retention volumes. The elution volumes of the benzodiazepines were determined using authentic specimens.

Table 1. *Retention volumes and detection limits for benzodiazepines and metabolites using reverse-phase liquid chromatography.*

Benzodiazepine	Retention vol (ml)	Sensitivity of detection (ng) λ254 nm†	λmax in column eluent‡ (nm)
2-Amino-5-chloro benzophenone	9·6	5·0	2·5 (238)
Chlordiazepoxide	6·5	4·0	4·0 (263)
Demoxepam	3·25	4·5	2·0 (238)
Desmethyldiazepam	5·9	5·0	2·5 (228)
Diazepam	8·1	5·0	2·5 (232)
Flurazepam	+8·8	9·0	6·0 (230)
Lorazepam	4·2	5·0	2·5 (225)
Medazepam	*10·9	4·0	2·5 (236)
2-Methylamino-5-chloro-benzophenone	*7·5	8·0	5·0 (225)
Nitrazepam	3·65	2·5	1·5 (220)
Oxazepam	4·3	4·0	2·0 (229)

Benzodiazepines were chromatographed on a column of Spherisorb-5-ODS (150 mm × 4·6 mm i.d.). The column eluent contained 60% methanol (v/v) in phosphate buffer (pH 7·8) at a flow rate of 1 ml min.
* 70% methanol (v/v) in phosphate buffer (pH 7·8); 1 ml min^{-1}.
\+ 80% methanol (v/v) in phosphate buffer (pH 7·8); 2 ml min^{-1}.
† Signal to noise ratio 8:1 ‡ Signal to noise ratio 5:1.

RESULTS AND DISCUSSION

The combined enzymic-h.p.l.c. method has been used on three types of sample: (a) human liver spiked with benzodiazepines at concentrations, of 10 $\mu g\ g^{-1}$; (b) guinea-pig livers containing benzodiazepines; (c) human liver from case samples obtained after suspected benzodiazepine poisoning.

(a) *Analysis of spiked human liver samples.* Direct extraction of benzodiazepines from tissue gives a poor recovery (Kaplan & others, 1973): thus simple ether extraction of liver to which had been added chlordiazepoxide, nitrazepam and flurazepam yielded 9, 10 and 18% recoveries respectively by conventional methods of drug release such as those described by Clifford & Franklin Smythe (1974). Even lower yields would be expected from post-mortem samples because of metabolism and increased drug-protein binding. Subtilisin enzymic digestion of tissues before extraction gave greatly enhanced recoveries over a wide range of concentrations (pg-mg) i.e. chlordiazepoxide 61, nitrazepam 66 and flurazepam 89%.

(b) *Analysis of liver from poisoned guinea-pigs.* The enzymic hydrolysis of liver samples from guinea-pigs administered with each of the benzodiazepines yielded extracts containing the drug and its metabolites in all cases except potassium clorazepate. The liquid chromatographic analysis described resolved adequately all of the benzodiazepines and their metabolites (Table 1). Co-extracted liver constituents present in extracts from drug-free livers were eluted from the column before the appearance of any of the drugs examined and thus did not interfere.

Diazepam is metabolized rapidly to desmethyldiazepam which is then slowly converted into oxazepam (Van der Kleijn, van Rossum & others, 1971; Bianchi, Fennesy & others, 1974). This accounts for our finding that in cases of diazepam poisoning the concentration of the parent drug is frequently low in comparison with that of desmethyldiazepam. (Typical concentrations were diazepam 3, desmethyldiazepam 64, oxazepam 9 $\mu g\ g^{-1}$.)

To obviate lengthy chromatographic analysis time (>15 min), stronger h.p.l.c. eluents were used for separations involving medazepam and flurazepam. A chromatogram of an enzyme hydrolysed liver extract after medazepam poisoning is shown in Fig. 1. The metabolites *N*-desmethyldiazepam (DMD), 2-amino-5-chlorobenzophenone (ACB), 2-methylamino-5-chlorobenzophenone (MACB) and oxazepam were present in addition to the parent drug. Typical analysis figures were: medazepam 20, DMD 35, ACB 6, MACB 13 and oxazepam 4 $\mu g\ g^{-1}$.

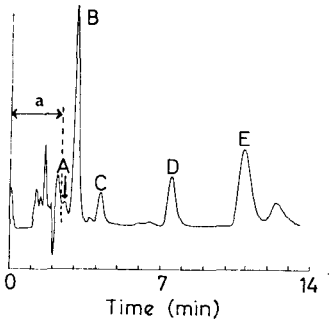

FIG. 1. H.p.l.c. of guinea-pig liver extract after administration of medazepam. Eluent: 70% methanol, pH 7·8; flow rate 1 ml min^{-1}. A = Oxazepam; B = *N*-desmethyldiazepam; C = 2-amino-5-chlorobenzophenone; D = 2-methylamino-5-chlorobenzophenone; E = medazepam. a—Peaks from control liver.

Potassium clorazepate was extracted from 'spiked' liver samples without difficulty, but only metabolites were detected in tissues of animals to which this drug had been administered. From a guinea-pig liver 110 μg g^{-1} of desmethyldiazepam and 8 μg g^{-1} oxazepam were recovered. Control experiments demonstrated that potassium clorazepate is unstable in the presence of even dilute hydrochloric acid. Reaction between 15 mg clorazepate and 10·0 ml hydrochloric acid (1·15 M, stomach concentration) resulted in the conversion of 84% of the drug into desmethyldiazepam after only 1 min, and after 12 min decomposition was complete at room temperature. It is unlikely that potassium clorazepate would be detected in tissues following oral ingestion.

Table 2. *Concentrations of benzodiazepines measured in cases of human self-poisoning.*

Case	Benzodiazepine present	Drug concn (μg g^{-1}) Subtilisin method	Acid hydrolysis
A	Flurazepam	51·2	37·5*
	Diazepam	9·5	4·2
B	Diazepam	2·8	0·9*
	Desmethyldiazepam	1·4	
C	Chlordiazepoxide	3·6	0·2
D	Diazepam	5·3	0·6
	Desmethyldiazepam	0·5	
E	Diazepam	21·5	1·2
	Desmethyldiazepam	1·2	
F	Desmethyldiazepam	7·3	0·0
G	Oxazepam	9·7	0·4
H	Oxazepam	13·2	2·0
	Desmethyldiazepam	80·6	
	Flurazepam	0·9	

All concentrations measured using the subtilisin method were unchanged benzodiazepines. Results of the acid hydrolysis method relate to benzophenones except in case A where unchanged benzodiazepines were measured.

* Acid hydrolysis concentrations for cases A & B were provided by A. Hoole (Nottingham Forensic Science Laboratory) and M. J. Bailey (Chorley Forensic Science Laboratory) respectively.

(c) *Analysis of human liver from suspected fatal poisoning cases.* Liver samples have been examined from eight suspected cases of benzodiazepine poisoning. Of the non-benzodiazepine drugs also present, viz amitriptyline, pethidine, imipramine, chlorpromazine and barbiturates, none interfered in the h.p.l.c. analysis of benzodiazepines.

Table 2 shows that for these eight cases, use of the proteolytic enzyme to release drugs from tissue proteins enhanced the yield of benzodiazepines.

In the liquid chromatographic method used, on-column decomposition of drugs was not encountered and interference from ultraviolet-absorbing impurities co-extracted from liver tissue was eliminated.

The retention volumes and sensitivity of detection at 254 nm for the drugs are given in Table 1 together with the sensitivity at λmax.

CONCLUSIONS

The bacterial proteolytic enzyme subtilisin Carlsberg has been used to liberate chemically labile and tightly protein-bound benzodiazepine drugs and their metabolites from animal and human tissues in far greater yields than the more classical methods of drug extraction. The method is simple, rapid and inexpensive, and offers the following advantages: (a) conditions involving strong mineral acids and high temperatures, known to be responsible for chemical degradation of benzodiazepines, are avoided; (b) the enzyme hydrolysed liver may be directly extracted with organic solvents without the risk of emulsion formation; (c) qualitative and quantitative analysis of solvent extracts may be performed rapidly by reverse-phase liquid chromatography, without elaborate clean-up procedures. Benzodiazepines and metabolites detectable at nanogram concentrations are well separated from each other and from co-extracted liver components.

The enzymic hydrolysis method can be used to extract benzodiazepines from blood, and to the separation of other classes of drugs from blood and tissue.

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