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DETECTION AND DETERMINATION OF COMMON BENZODIAZEPINES AND THEIR METABOLITES IN BLOOD SAMPLES OF FORENSIC SCIENCE INTEREST

MICROCOLUMN CLEANUP AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH REDUCTIVE ELECTROCHEMICAL DETECTION AT A PENDENT MERCURY DROP ELECTRODE

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SUMMARY

Benzodiazepines in the blood samples typical of forensic science work are recovered from 100–250 μl amounts of blood (diluted with aqueous sodium octyl sulphate to suppress protein binding) onto microcolumns of Porapak-T, and finally eluted into 60-μl volumes of aqueous acetonitrile. The eluates may be taken directly for analysis by high-performance liquid chromatography (HPLC) with reductive amperometric detection at a pendent mercury drop electrode held at potentials down to -1.2 V *vs.* Ag/AgCl. For high sensitivity work the electrode is preceded by a coulometric detector fitted with porous carbon electrodes held at 0 V (proprietary reference electrode). The technique detects all of the commonly encountered benzodiazepines and others except clobazam, which contains no azomethine group. The detection limits generally are in the range 1–5 ng/ml (40–200 pg HPLC-injected) in haemolyzed human blood, with recovery values of 84–95%, depending on the actual benzodiazepine, over the range examined ($\leqslant 2.14$ μg/ml). The respective values for the metabolites of nitrazepam are 8–12 ng/ml and 75–84%. The technique is very much less susceptible to the interferences afflicting other commonly applied techniques, and facilitates considerably the analysis of degraded samples.

INTRODUCTION

Although many techniques have become available for the analysis of benzodiazepines and their metabolites in body fluids[1–4], difficulties remain in the detection and quantitation of these compounds in the blood samples routinely encountered in forensic science casework. Such samples are varyingly and usually extensively haemolyzed and are often putrified. Large amounts of solidified and coagulated materials may be present, and there may be serious contamination by plasticizers and by components of the elastomers, *e.g.* in rubber septa, that the samples may contact

during their collection and storage. The quantities of blood available for examination may be small, and further restricted by the requirements of other analyses.

Usually the samples are examined first by radioimmunoassay techniques that are broadly-specific to this group of compounds[5]. Any cross-reactivity is then subject to identification by other techniques, which commonly depend on chromatography in some form, and in turn depend considerably for their effectiveness on an efficient sample-preparation procedure. Until recently, as in clinical practice, liquid–liquid extraction procedures have predominated[6–9], but there are obvious possibilities in the use of the solid-phase extraction techniques that are well established in the analysis of the more tractable samples encountered clinically[10–15].

In forensic science work, as elsewhere, the application of solid-phase extraction has been promoted by the commercial availability of prepacked column and cartridge extraction assemblies. We are aware of no published applications, forensically, to the benzodiazepines, but applications have been reported in the detection of morphine[16], morphine and codeine[17], and several anti-inflamatory drugs[18]. Given a sufficient amount of undegraded sample these applications are of undoubted value, but problems emerge with small and degraded samples. In our experience the assemblies tend to become blocked (although a recently developed technique apparently prevents this[19]), and the relatively large amount of adsorbent often present necessitates a final elution either with a large volume of a weak eluent and consequent excessive dilution, or with a strong eluent and consequent poor selectivity.

The small-scale transfer of the benzodiazepines into microlitre volumes can be made by solid-phase extraction onto a loose adsorbent, after liquid–liquid extraction and evaporation steps[20]; and triazolam has been extracted from *post mortem* samples by the direct addition of loose adsorbent[21]. Both techniques give the non-aqueous solutions required for the subsequent gas chromatography. However, if reversed-phase liquid chromatography can be used there is no need for transfer to non-aqueous solvents, and advantage can be taken of the superior stability of the benzodiazepines under liquid chromatography conditions. For the satisfactory gas chromatography of some benzodiazepines derivatization may be required[20].

Recently, for use in conjunction with reversed-phase liquid chromatography, microcolumn cleanup techniques have been applied in the examination of small and highly complex samples for traces of explosives and firearms residues. A summary of most of this work is available[22]. As described here these techniques have now been adapted to the examination of blood samples for benzodiazepines. The manipulation is straightforward, and with a throughput time limited by the chromatographic run-time, which is within 10 min for all of the commonly encountered benzodiazepines.

Most of the published work on the liquid chromatography of the benzodiazepines has employed ultraviolet absorbance detection; but nearly all of these compounds are electrochemically reducible at practicable electrode potentials[1,23,24]. and high-performance liquid chromatographic (HPLC) techniques employing the effect have been described[25–27]. Despite this, for only those compounds carrying very readily reduced substituents has electrochemical detection given sensitivities competitive with those from ultraviolet absorbance, mainly because of the interference caused by the reduction of traces of oxygen dissolved in the eluents. To a lesser extent, difficulties have arisen due to contamination and irreproducibility of some working electrodes, and to the noise levels generated by dropping mercury electrodes. However,

following from the work on explosives traces none of these problems remains of significance[28], and reductive mode detection employing a pendent mercury drop electrode is now a routinely applied technique in this laboratory. The electrode response characteristics are highly reproducible (from one year to the next), and the technique is as easily used as any other offering parts-per-billion sensitivity. The application to the common benzodiazepines and their metabolites at these sensitivity levels is described in the following.

EXPERIMENTAL

Materials

Solvents are HPLC grade (Rathburn); disodium hydrogenphosphate and potassium dihydrogenphosphate are Aristar grade (BDH). Sodium octyl sulphate is HPLC grade (Eastman Kodak).

The Porapak-T (Millipore-Waters), 75–100 μm, is Soxhlet-extracted with acetonitrile for 8 h and dried, finally, under vacuum at room temperature. At monthly intervals the adsorbent is washed with acetonitrile at a filter pump and vacuum-dried.

The benzodiazepines are reference compounds provided by the Central Research Establishment, Home Office Forensic Science Service, U.K. The 6-nitroquinoline (98%, Aldrich) gives a single chromatography peak at usual sensitivity levels.

Blood samples are equine blood stabilized with oxalate (Gibco); and human blood, stabilized with citrate, from rejected transfusion bags (National Blood Transfusion Service, U.K.). Other samples, variously stabilized, are from routine casework. To haemolyze them, samples are frozen and thawed.

HPLC equipment and conditions

The essential details are as in the work on the electrochemical detection of explosives, recently summarized[28]. Briefly, the detector (EG&G Brookdeal, Model 310, with the 174A control unit) is a modified hanging mercury drop electrode maintained usually at −1.2 V *vs.* Ag/AgCl (0.5 *M* aq. lithium chloride); the electrode drop size is 6 mg, given by the "L" setting on the control unit; and the separation between the eluent jet and the tip of the mercury capillary is 0.7 mm. The only routine maintenance of the electrode unit is the occasional reinstallation of a freshly silanized glass capillary. The silanization procedure given in the manufacturer's manual is followed with one important modification. After the glass surface has been cleaned by washes in turn with aqueous hydrofluoric acid and water, the treatment is repeated with 0.2% (w/v) aqueous sodium hydroxide and water before the capillary is dried and silanized. Such capillaries give *ca.* six months use before instability of the mercury drop necessitates their replacement.

On occasions, either or both an ultraviolet (240 nm) absorbance detector (Pye Unicam, LC–UV detector) and a coulometric detector (Environmental Sciences Associates, Model 5100A Coulochem with a 5010 analytical cell) have been inserted upstream of the mercury electrode. The coulometric detector, with both of its porous carbon working electrodes set at 0 V (proprietary reference electrode), enables the background current at the mercury electrode to be appreciably reduced in work at high sensitivities.

The separations of the common benzodiazepines are made on 150 × 4.5 mm columns of ODS-Hypersil (Shandon), 3 μm, at 40°C. The eluent, 1 ml/min, is methanol–1-propanol–aqueous phosphate (100:7.5:80, v/v/v), where the aqueous phosphate (pH 6.0) is 0.0174 *M* in potassium dihydrogenphosphate and 0.0026 *M* in disodium hydrogenphosphate. The injection volume of the deoxygenated samples[29] is 10 μl. Some less common and more strongly retained benzodiazepines can be separated rapidly on a 100 × 4.5 mm column of CPS-Hypersil (Shandon) under otherwise unaltered conditions. Along with the other straightforward precautions necessary to exclude oxygen[28] the eluent reservoir is kept under a gentle reflux when the chromatograph is in use. The often recommended vigorous purging of the eluent with an inert gas is quite unnecessary, although the introduction of a low gas flow (*ca.* 5 ml/min) through a porous frit in the eluent is essential to the establishment of a smooth reflux.

Extraction and cleanup equipment and reagents

The sodium octyl sulphate is made up to 0.2% (w/v) in aqueous 0.1 *M* disodium hydrogenphosphate. The microcolumn eluents are acetonitrile–water [7:4 and 1:8 (v/v), subject to the retentivity of the batch of Porapak-T used].

Centrifugal filters are made from 1.5-ml polypropylene centrifuge tubes. A hole, *ca.* 1 mm, is pierced in the apex of a tube, and 10–20 mg of Soxhlet-extracted (methanol, 8 h) cotton wool is pushed firmly down into the apex. An unmodified tube is used as a receiver. Disposable microcolumns of 5 mg of the Porapak-T in 60 mm × 1 mm I.D. PTFE tubing (Alltech) are prepared as otherwise previously described[30]. At this stage only about one third of the column tube is filled. The columns may be stored indefinitely, but immediately before use 50–100 μl of acetonitrile and then a similar volume of water is drawn through them at a filter pump. Membrane microfilters, 0.45-μm pore size PTFE (Gelman, Acro LC3S), and 250-μl polypropylene microcentrifuge tubes (Alpha Laboratories) are Soxhlet-extracted (methanol, 8 h) and dried ⩽80°C.

The elution equipment is a chromatography injection valve (Rheodyne, Model 7125) fitted with a 1-ml sample loop that serves as a reservoir for one of the eluents. The valve is connected to a conventional HPLC pump, the inlet flow lines of which have been replaced with 1 mm I.D. stainless-steel tubing to minimize the internal volume of the equipment. The pump is charged with the other of the eluents and set to a flow of 1 μl/s. A timing unit controls the pump. For 60-μl fractions the system gives a coefficient of variation of 4%. At this low flow-rate bubble formation in the pump chambers can be troublesome, but is readily avoided if the eluents are sonicated before use. (The back pressure of the columns is negligible: any type of pump could be used, or the columns could be eluted manually from a syringe.)

Sample cleanup and processing procedures

A flowchart summarizing these procedures is shown in Fig. 1.

The sample is spun through one of the centrifugal filters. This removes coarse particulates and coagulated material; cellular material passes through. The filtrate is mixed and a portion, ⩽250 μl, is transferred to a 1.5-ml polypropylene centrifuge tube. A sample size of 100 μl is sufficient in most cases. If the volume of unfilterable material can be disregarded, the actual quantity of blood required for analysis may be pipetted

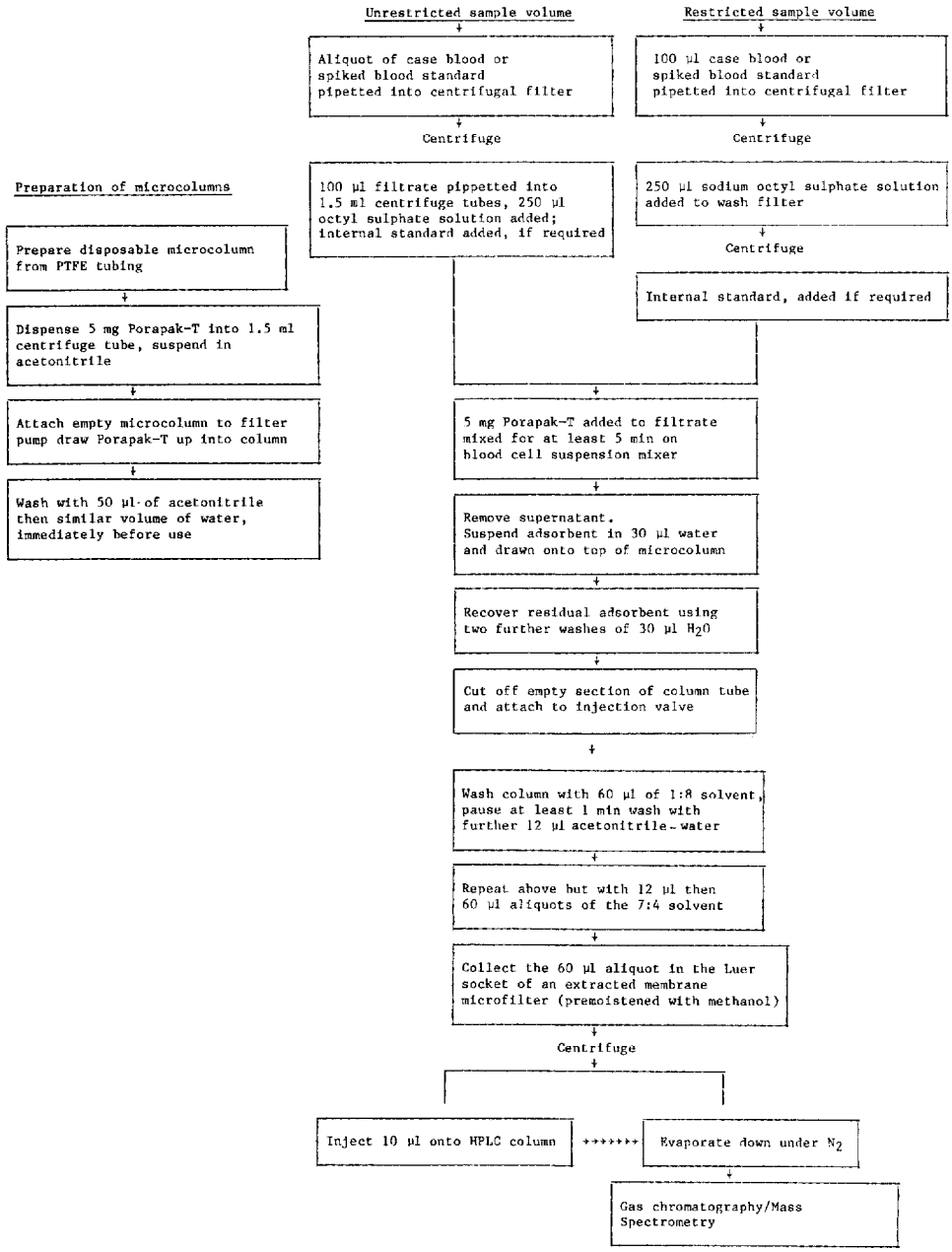

Fig. 1. Sample preparation and processing: schematic flowchart representation of the details given in Experimental.

directly onto the filter and washed through at the centrifuge with the next added reagent. This minimizes sample wastage on the filter.

Into the sample is dispended 250 µl of the octyl sulphate solution and 5 mg of the Porapak-T. The mixture is kept in suspension for not less than 5 min on a blood cell

suspension mixer, the adsorbent is allowed to settle out for a similar time, and the supernatant is removed. The adsorbent is drawn onto the top of a microcolumn (attached at its outlet to a filter pump) with three 30-μl volumes of water, the section of the column tube remaining empty is cut off, and the column is attached to the injection valve. Elution is conducted first with 60 μl of the 1:8 solvent. After a pause of not less than 1 min, when a slowly desorbing blood pigment is released, the elution is continued in turn with 12 μl of the same solvent (12 μl is the approximate column void), and with 12 μl and 60 μl increments of the 7:4 solvent. The benzodiazepines appear in the last increment. This is collected in the Luer socket of an extracted membrane microfilter (premoistened with methanol), and centrifuged through the membrane into a micro-centrifuge tube. The centrifugate is taken for chromatography directly. If necessary, part of it may be evaporated down in a stream of nitrogen and transferred to any solvent appropriate, *e.g.*, to gas chromatography or mass spectrometry.

Quantitative determinations are standardized against spiked blood samples. Internal standards, in aqueous methanol, may be added directly to the samples or to the octyl sulphate solution. For general-purpose work the 6-nitroquinoline added at 2–20 ng per sample is convenient. If a benzodiazepine is used the addition is made directly into the sample: the alternative mode can result in losses due to precipitation and adsorption of the less polar benzodiazepines.

TABLE I

HYDRODYNAMIC VOLTAMMETRIC RESPONSE DATA OF SOME BENZODIAZEPINES, THEIR METABOLITES, AND 6-NITROQUINOLINE UNDER HPLC CONDITIONS

The data are from peak area measurements.

Compound	*Response**		
	Relative to equimolar 6-nitroquinoline, at −1.2 V	*Relative to the individual compounds' responses at −1.2 V*	
		−1.1 V	*−1.0 V*
7-Acetamidonitrazepam**	0.34	0.13	<0.05
7-Aminonitrazepam**	0.30	0.05	<0.05
Chlordiazepoxide	0.68	1.00	0.70
Demoxepam**	0.72	0.67	0.49
Desmethylchlordiazepoxide**	0.52***	0.90	0.81
Desmethyldiazepam**	0.27	0.33	<0.05
Diazepam	0.34	0.36	<0.05
Loprazolam	1.23	0.82	0.48
Lorazepam	0.30	0.45	<0.05
Lormetazepam	0.27	0.20	<0.05
Nitrazepam	0.97	0.74	0.69
6-Nitroquinoline	(1.00)	0.79	0.75
Oxazepam	0.35	0.23	<0.05
Temazepam	0.32	0.10	<0.05
Triazolam	0.38	0.98	0.44

* Means of duplicated determinations. The maximum difference within a pair of duplicates was 0.08 (desmethylchlordiazepoxide at −1.1 V).

** Metabolites.

*** Impurity peaks present.

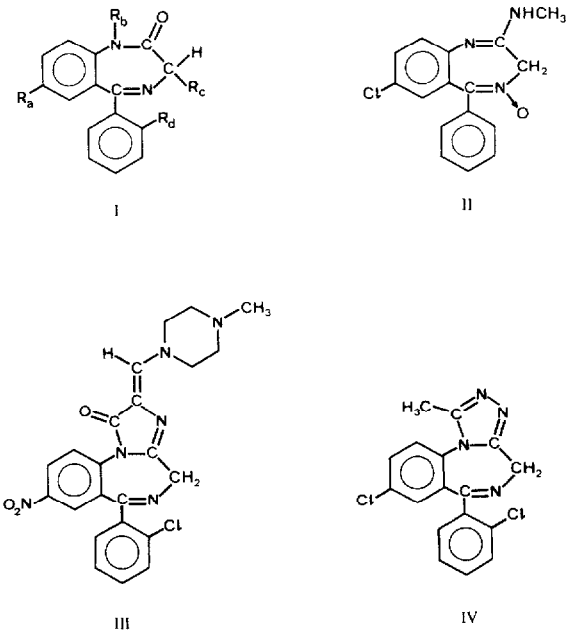

Fig. 2. Structural formulae of common benzodiazepines and their metabolites: 7-acetamidonitrazepam (I, R_a = CH_3CONH, $R_b = R_c = R_d = H$); 7-aminonitrazepam (I, R_a = NH_2, $R_b = R_c = R_d = H$); chlordiazepoxide (II); demoxepam (II, the desmethylamino lactam); desmethylchlordiazepoxide (II, the desmethyl analogue); desmethyldiazepam (I, R_a = Cl, $R_b = R_c = R_d = H$); diazepam (I, R_a = Cl, R_b = CH_3, $R_c = R_d = H$); loprazolam (III); lorazepam (I, R_a = Cl, R_b = H, R_c = OH, R_d = Cl); lormetazepam (I, R_a = Cl, R_b = CH_3, R_c = OH, R_d = Cl); nitrazepam (I, R_a = NO_2, $R_b = R_c = R_d$ = H); oxazepam (I, R_a = Cl, R_b = H, R_c = OH, R_d = H); temazepam (I, R_a = Cl, R_b = CH_3, R_c = OH, R_d = H); triazolam (IV).

RESULTS AND DISCUSSION

The work has been concerned primarily with the benzodiazepines approved for supply on a British National Health Service prescription —the "limited list". Those examined in detail, together with some metabolites of them, are listed in Table I; structural formulae are given in Fig. 2 (comprehensive listings are available in the cited literature[2,3]). Except for clonazepam all of the benzodiazepines encountered during the period of development and application of the described techniques (14 months; 87 cases, 64 positive) are included in the table. Clonazepam (1 case) and the benzodiazepines available on private prescription have been less closely examined, but with the result that the described work is equally applicable to them without significant modification. The only exception is clobazam, which is not amenable to reductive detection and has not been involved in any submitted casework.

Electrochemical characteristics

Except for that of clobazam, the characteristic diazepine ring of the compounds contains an azomethine bond (Fig. 2) reducible at accessible potentials on a mercury electrode[23]. More readily reduced nitro, amine oxide and heterocyclic functions occasionally are present, which confers detectability at carbon electrodes[25,31]. However, the mercury electrode is applicable generally.

Some reponse characteristics under the HPLC conditions of the compounds of interest are given in Table I. At −1.2 V, *vs.* Ag/AgCl, the data are expressed relative to 6-nitroquinoline. To a first approximation, they may be interpreted on the assumption that azomethine, amine oxide and nitro groups are undergoing, respectively, their 2-, 2- and 4-electron reductions to the secondary amine and to the incompletely reduced derivatives of the last two groups[32,33]. There is no evidence of the reductive dehydroxylation undergone by the hydroxybenzodiazepines under some conditions[34]. In loprazolam the response is probably augmented by reduction processes within this nitrocompound's more complex heterocyclic structure. There seems to be a similar participation in the case of triazolam where, as Table I shows, at −1.0 V a substantial response persists relative to this compound's response at −1.2 V, in contrast to the solely azomethine reductions (relative responses <0.05). Presumably, reduction within the ring structure of the nitroquinoline, along with reduction of the nitro group, is responsible for the coincidence between the nitroquinoline and nitrazepam at −1.2 V.

For work with nanogram amounts the potential limit, because of baseline drift, is in the region of −1.4 V. At this potential a substantially increased response is obtained from the amine oxide- and nitro-substituted benzodiazepines, but not from the others. The result is expected on the above assumption that at −1.2 V only the azomethine reductions are complete.

Background currents

It has often been suggested that reductive detection techniques are difficult to use because of the requirement for a deoxygenated eluent if a low background current and, hence, a stable baseline and a satisfactory sensitivity are to be obtained. However, there should be no difficulty if the eluent reservoir is maintained under a slow reflux whilst the system is in use, if all of the eluent flow lines are of stainless steel, and if the eluent flow is stopped completely when the system is not in use[28]. Some years ago it was demonstrated on the basis of ultraviolet absorbance results that for practical HPLC use the reflux technique is much more effective than the commonly employed sparging technique[35]; and in view of our own results and those available from a variety of other techniques, which have been reviewed recently[36], this remains the position.

For the detection of the benzodiazepines down to the 50 ng/ml level the reflux technique is all that is necessary, and results in background currents of 5–20 nA, cathodic (chromatographic peak heights are in the range 0.25–2.5 nA per ng of benzodiazepine injected). Aerated eluents give background currents of *ca.* 20 μA.

The installation of the upstream coulometric detector with both of the porous carbon working electrodes at 0 V (proprietary reference electrode) lowers the background current at the mercury electrode to 2–6 nA, cathodic; and with a regularly used system a stable baseline is obtained after a short running time, *e.g.*, 10 min. Without this installation an hour's running time may be needed. Potentials at the carbon electrodes more cathodic than 0 V effect little further depression of the background current. The substances undergoing reduction at the carbon electrodes are unknown, but traces of oxygen may not be involved: at 0 V oxygen is not significantly reduced on this type of electrode[37], and the electrodes proved ineffective in attempts to depress the response of the mercury electrode to oxygenated eluents. The carbon electrodes operated at negative potentials, *e.g.* −0.7 V, to some extent respond to the

readily reduced benzodiazepines, but the response is inefficient and noisy, and of little use for nanogram amounts relative to the mercury electrode.

HPLC conditions

The general order of elution of the benzodiazepines under the present conditions agrees with the relevant published data from octadecylsilyl columns in aqueous methanolic eluents[38]. However, to display distinguishable peaks from all of the benzodiazepines of interest and to separate them from occasionally seen contaminants on a single chromatogram, the addition of 1-propanol to the eluent is necessary. The chromatogram of a standard mixture is shown in Fig. 3. If 1-propanol is not present, triazolam, lorazepam and oxazepam are unresolved from one another. So too are chlordiazepoxide and lormetazepam. Under the conditions of Fig. 3 desmethylchlordiazepoxide (not included in the mixture) is unresolved from lorazepam, but readily distinguished by the voltammetric response ratios (Table I); and clonazepam overlaps nitrazepam, although the individual retention times are distinguishable with

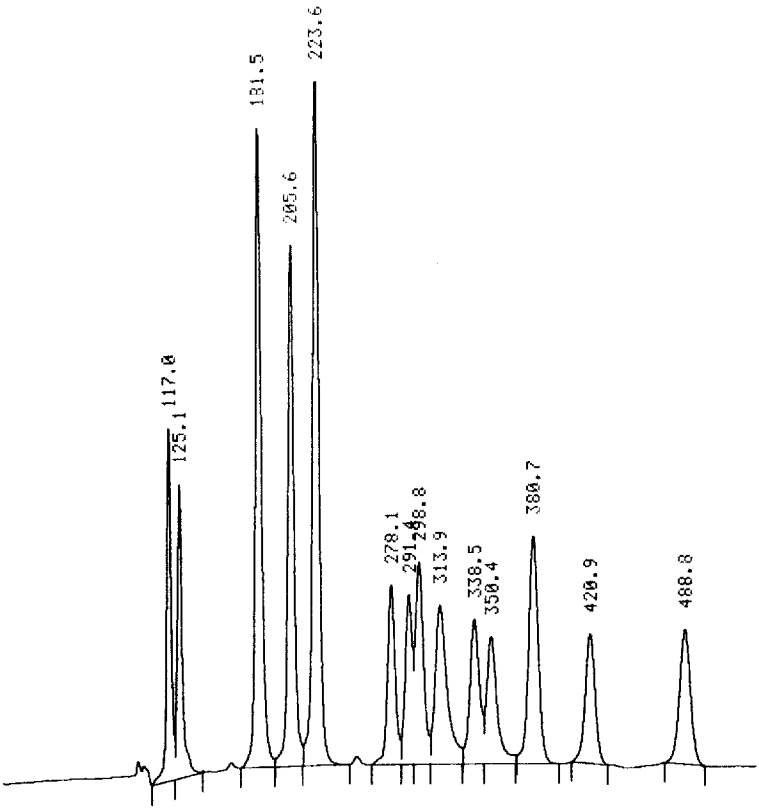

Fig. 3. Reductively detected liquid chromatogram of a mixture of standard compounds (5–10 ng) under the conditions given in Experimental. The retention times (s) are superscripted on the peaks. Identities in order of elution are: 7-aminonitrazepam (117.0 s), 7-acetamidonitrazepam (125.1 s), 6-nitroquinoline (181.5 s), demoxepam (205.6 s), nitrazepam (223.6 s), triazolam (278.1 s), lorazepam (291.4 s), oxazepam (298.8 s), loprazolam (313.9 s), temazepam (338.5 s), lormetazepam (350.4 s), chlordiazepoxide (380.7 s), desmethyldiazepam (420.9 s), diazepam (488.8 s). The maximum peak amplitude is 20 nA.

clonazepam running slightly ahead. The pair may be resolved if the propanol is omitted from the eluent. As the column ages loprazolam is increasingly retained relative to the other benzodiazepines, probably because of the interaction of this comparatively basic compound with developing active sites. The effect may be countered by increases in the propanol content of the eluent, *e.g.*, by increments of 10% in the propanol present. Probably greater modification would be necessary to adapt the eluent to different batches of the column packing. Except for the switching to the cyanopropyl-bonded column, unmodified conditions may be used for the more strongly retained compounds such as medazepam and flurazepam.

Cleanup procedure

The various adsorbents and ion exchange resins investigated include the Porapaks -Q, -R, -S and -T (Millipore-Waters), Chromosorb-104 (Johns-Manville), Amberlite XAD-4 and Amberlyst-15 (Rohm and Haas), and Partisil-SCX (Whatman). The last two are ion exchange materials that, in the hydrogen form, gave excellent recoveries from blood of all of the benzodiazepines except triazolam, most of which apparently decomposed. Of the other adsorbents, Porapak-T proved to be the most satisfactory in terms of the cleanliness and efficiency of the extractions, and of the convenience of its use. The relatively high density of this adsorbent promotes clean sedimentation from its mixtures with diluted blood.

From aqueous solutions the values of the distribution ratios for the adsorption of benzodiazepines on Porapak-T are in the range 1000–2000 ml/g. In blood samples, however, the extent of adsorption sometimes is substantially less than these values require. The effect occurs most strongly in transfusion bloods, and negligibly in degraded samples and in samples of equine blood, and can be supressed by the addition of fatty acids, and by sodium octyl and dodecyl sulphates. Diazepam and temazepam are the most strongly affected. Presumably adsorption on the Porapak is in competition with binding to serum albumin; diazepam and temazepam are known to be relatively strongly bound[39]; fatty acids compete with benzodiazepines for binding sites on serum albumin[40]; the binding varies widely between samples[41]; and the structure of serum albumin is disrupted by alkyl sulphates[42]. Sodium octyl sulphate is used to suppress the binding in preference to the dodecyl sulphate particularly because the dodecyl sulphate tends to retain temazepam specifically in solution, probably within micelles. Evidently, in many published extraction techniques the binding may be ignored. The need to suppress it here arises from the use of a small proportion of the extractant to the sample.

The transfer of the loose adsorbent from the sample to the top of a prepacked microcolumn minimizes loss of the less strongly retained compounds (triazolam and the nitrazepam metabolites) during the transfer and subsequent washing. Relatively low concentrations of acetonitrile effect the desorption, which provides, therefore, a small volume of substantially aqueous extract that may be used for HPLC directly. The optimal concentration of acetonitrile may vary between batches of Porapak-T, however. Of three batches examined, the two with which the work described has been conducted were indistinguishable, but the other was appreciably more retentive.

Detection limits and recoveries

In Table II are given detection limits obtained from spiked, haemolyzed

TABLE II

DETECTION LIMITS AND REPEATABILITY OF RECOVERIES OF SOME BENZODIAZEPINES AND THEIR METABOLITES FROM HAEMOLYZED HUMAN BLOOD

Compound	*Detection limit* (ng/ml)*	*Mean recovered concentration** (ng/ml)*	
		Spiked at 66.9 ng/ml	*Spiked at 2140 ng/ml*
7-Acetamidonitrazepam	7.8	56.8 (2.96)	2324 (45.3)
7-Aminonitrazepam	12.4	55.9 (1.85)	1638 (25.1)
Chlordiazepoxide HCl	1.5	59.5 (1.43)	1973 (41.1)
Demoxepam	1.1	59.5 (1.73)	2074 (38.3)
Desmethyldiazepam	2.1	57.2 (2.28)	1889 (48.8)
Diazepam	1.8	56.4 (0.68)	1827 (65.7)
Loprazolam	2.1	63.2 (1.28)	2021 (47.9)
Lorazepam	1.1	57.7 (1.98)	1916 (45.2)
Lormetazepam	3.1	56.5 (1.72)	1901 (50.0)
Nitrazepam	0.32	30.5 (0.60)***	1066 (25.3)***
Oxazepam	1.8	57.0 (1.91)	1905 (44.8)
Temazepam	3.7	56.6 (1.51)	1865 (44.0)
Triazolam	2.0	64.0 (1.55)	2128 (50.3)

* Taken as three standard deviations of determinations ($n = 7$) in the range 1–5 ng/ml.
** $n = 6$; values in parentheses are standard deviations.
*** Half the indicated spike.

transfusion blood (in many techniques the plastics additives contaminating this material interfere). The detection limits are calculated as three standard deviations of the benzodiazepines recovered (expressed as concentrations in the blood) from 250-μl samples spiked in the range 1–5 ng/ml. Evidently, most of the detection limits lie in this range, corresponding to HPLC injections of 40–200 pg. For the majority of the benzodiazepines this is well below the therapeutic level although approached by some low dosage forms. Triazolam is an example, for which a chromatogram from the blood spiked at 5 ng/ml is given in Fig. 4A. The unspiked blood is shown in Fig. 4B, and exhibits no peaks beyond 150 s where most of the benzodiazepines occur. The electrode potential used here (-1.2 V) is considerably more cathodic than necessary. Triazolam may be detected readily at less negative potentials (Table I) with an approximate halving of the background current (at -1.0 V) and a corresponding improvement in the detection limit.

Included in Table II are repeatability data at concentrations well above the detection limits. The results are distributed with standard deviations corresponding to coefficients of variation in the range 1.2–5.2%. The upper limit is due to the acetamidonitrazepam, which is eluted in the early, congested region of the chromatograms.

Table III gives results from recovery experiments made on blood samples spiked with twofold increasing concentrations of up to 2.14 μg/ml. The nitroquinoline is included here because of its use in some of the work as an internal standard. Because of the extended concentration range taken, a variance-weighted least squares regression[43] was applied, with variance estimates derived from the data in Table II on the assumption that the standard deviations vary linearly with the magnitude of the recovered concentrations, and decrease to the limiting values used in the estimates of

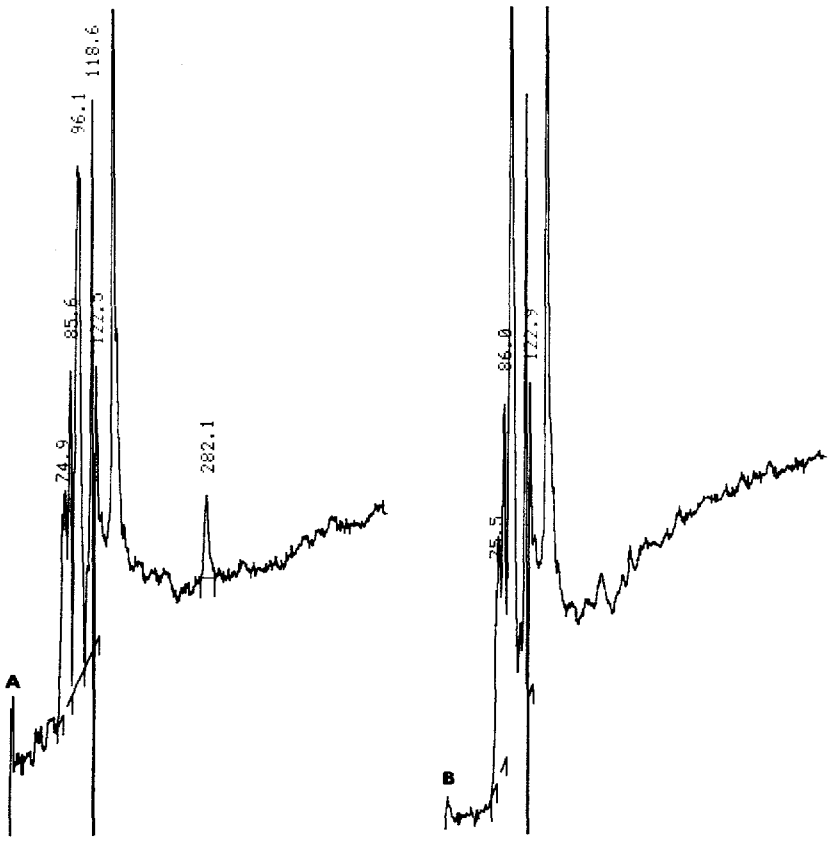

Fig. 4. Chromatograms of extracts from haemolyzed human blood: spiked with 5 ng/ml triazolam (A), and unspiked (B). The triazolam peak is at 282.1 s, with an amplitude of 80 pA (reductive detection).

the detection limits. Apart from the aminonitrazepam result, the data in Table III correspond to recoveries overall of 83.9% (oxazepam, acetamidonitrazepam) to 95.3% (loprazolam).

Results with reference to ultraviolet absorbance detection and other techniques

The generally lower recoveries (83.9%, 75.4%), and their greater variation, of the nitrazepam metabolites (Table III) might be improved under modified conditions, particularly by the use of more aqueous eluents, but at a cost of increased running times and poorer detection limits for the other benzodiazepines. Even so, the technique yields results previously obtained with considerable difficulty or not at all. In Fig. 5B is shown an example of a degraded blood sample submitted in a nitrazepam overdose case. Typically, no nitrazepam remained in the blood, but the chromatogram is dominated by an intense peak formed by 7-aminonitrazepam (121.9 s) at an estimated concentration of 600 ng/ml. The sometimes-detected acetamido metabolite is absent here. The only other strong peak is due to the internal standard (6-nitroquinoline). The immunoassay technique[5] is insensitive to nitrazepam metabolites even at this concentration. Also present in the sample were small amounts of desmethyldiazepam

TABLE III

RECOVERY EXPERIMENTS: LEAST SQUARES LINEAR REGRESSION (VARIANCE-WEIGHTED) OF CONCENTRATIONS FOUND ON CONCENTRATIONS ADDED TO HAEMOLYZED HUMAN BLOOD OF SOME BENZODIAZEPINES, THEIR METABOLITES, AND 6-NITROQUINOLINE

The regressions were parameterized with data included in Table II. None of the sets of recovery data varied significantly from linearity (runs test), or exhibited an intercept differing significantly from zero.

Compound	*Range (ng/ml)*	*n*	*Correlation coefficient*	*Slope (recovery ratio)*	*Standard error of slope*
7-Acetamidonitrazepam	16.7–2140	7	0.9988	0.839	0.018
7-Aminonitrazepam	16.7–2140	7	0.9966	0.754	0.028
Chlordiazepoxide	8.4–2140	9	0.9991	0.912	0.014
Demoxepam	8.4–2140	9	0.9993	0.948	0.014
Desmethyldiazepam	8.4–2140	9	0.9991	0.879	0.014
Diazepam	8.4–2140	9	0.9989	0.864	0.015
Loprazolam	8.4–2140	9	0.9990	0.953	0.016
Lorazepam	8.4–2140	9	0.9990	0.881	0.016
Lormetazepam	8.4–2140	9	0.9991	0.890	0.014
Nitrazepam	4.2–1070	9	0.9996	0.940	0.011
6-Nitroquinoline	2.1–535	9	0.9993	0.985	0.014
Oxazepam	8.4–2140	9	0.9986	0.839	0.017
Temazepam	8.4–2140	9	0.9989	0.873	0.015
Triazolam	8.4–2140	9	0.9993	0.872	0.014

and diazepam (60 and 20 ng/ml, respectively), as the chromatogram in Fig. 5B shows. The desmethyldiazepam could be detected, in line, by ultraviolet absorbance at 240 nm (Fig. 5A). This peak (431.3 s) has been used to scale the chromatogram to give a peak height equal to the same component in the electrochemically detected chromatogram. The comparison between the two demonstrates immediately the inferior selectivity of the ultraviolet detection. The aminonitrazepam peak, which should be similar in height to the peak in Fig. 5B (from experiments with the pure compounds), is entirely swamped by irrelevant material. Several such peaks are present, diazepam is undetectable, and the desmethyl compound is only a minor component of the chromatogram.

The result illustrated in Fig. 5 is representative of a number of comparisons that have been made. Out of 14 examples from casework, ultraviolet detection gave readily interpreted chromatograms in 6 cases. Of the remainder, 4 were too complex to be of any value, and 4 could be interpreted with reference to the electrochemically detected chromatograms. Evidently a commonly held view, formed without apparent reference to selectivity, that ultraviolet detection gives at least comparable if not superior sensitivity to reductive detection of the benzodiazepines carrying no readily reducible substituents, is not applicable to the type of samples involved here —the improved electrochemical sensitivity notwithstanding. It may be argued that the cleanup procedure is more suitable for the electrochemical technique. However, from our earlier use of liquid–liquid extractions and of solid phase extractions employing the commercially available cartridges, in conjunction with ultraviolet detection, far more complex extracts were obtained then than now. Also the present extracts are found to

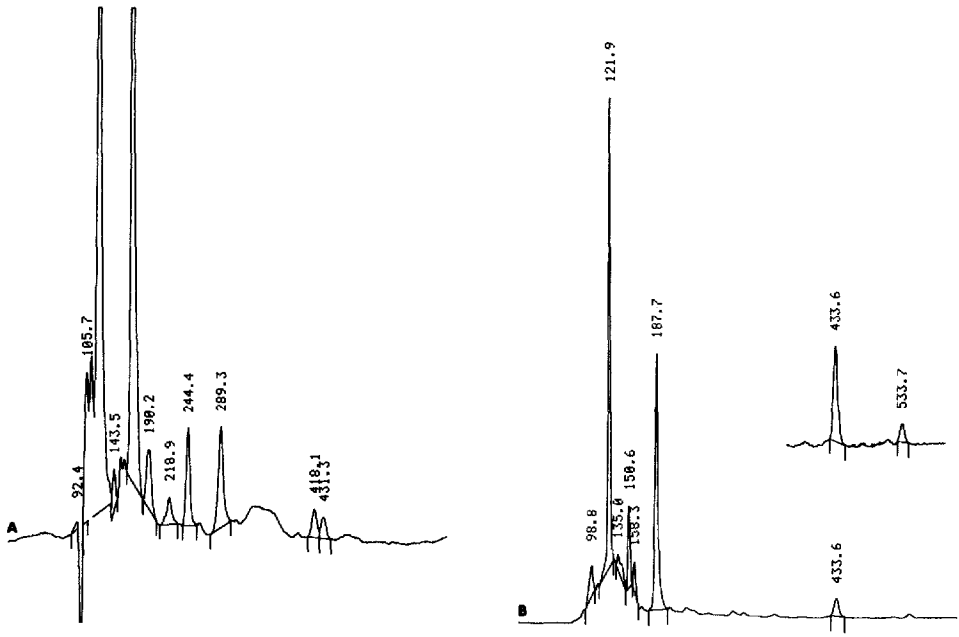

Fig. 5. Chromatogram of an extract of *post mortem* blood with ultraviolet absorbance detection at 240 nm (A) and reductive detection (B). The peak assignments (chromatogram B) are 7-aminonitrazepam (121.9 s), internal standard (187.7 s), desmethyldiazepam (433.6 s) and diazepam (533.7 s). The chromatograms are scaled to give similar peak heights for desmethyldiazepam (at 431.3 s in A), for which the approximate amplitudes are 0.0016 absorbance units (A), and 0.95 nA (B).

be far cleaner by gas chromatography with electron capture and mass spectrometric detection.

Oxidative detection at the porous carbon electrodes was briefly investigated. In agreement with the published results[44] a number of the benzodiazepines could be detected, but many interfering peaks were present in the chromatograms of casework samples.

Examples of "difficult" samples

Shown in Fig. 6 are chromatograms of three *post mortem* blood samples (A–C) from cases not involving benzodiazepines, and a chromatogram from a sample submitted in connection with a road traffic offence (D). All the chromatograms are scaled to the same sensitivity. Sample C was undegraded; A and B are examples of partly putrified material, and illustrate the insensitivity of the technique to the interferences due to decomposition products.

Sample D was obtained in a glass vial crimp-sealed with a rubber septum. The chromatogram includes peaks due to the internal standard (180.6 s), oxazepam (297.5 s) and temazepam (337.1 s). The respective concentrations of the benzodiazepines are 60 and 570 ng/ml. The other major peak (394.1 s) is characteristic of this particular make of septum, and is an extreme example of an occasionally encountered contamination. Possibly a sulphur-containing compound is responsible. No interference occurs with any of the benzodiazepines, all of which are well separated from

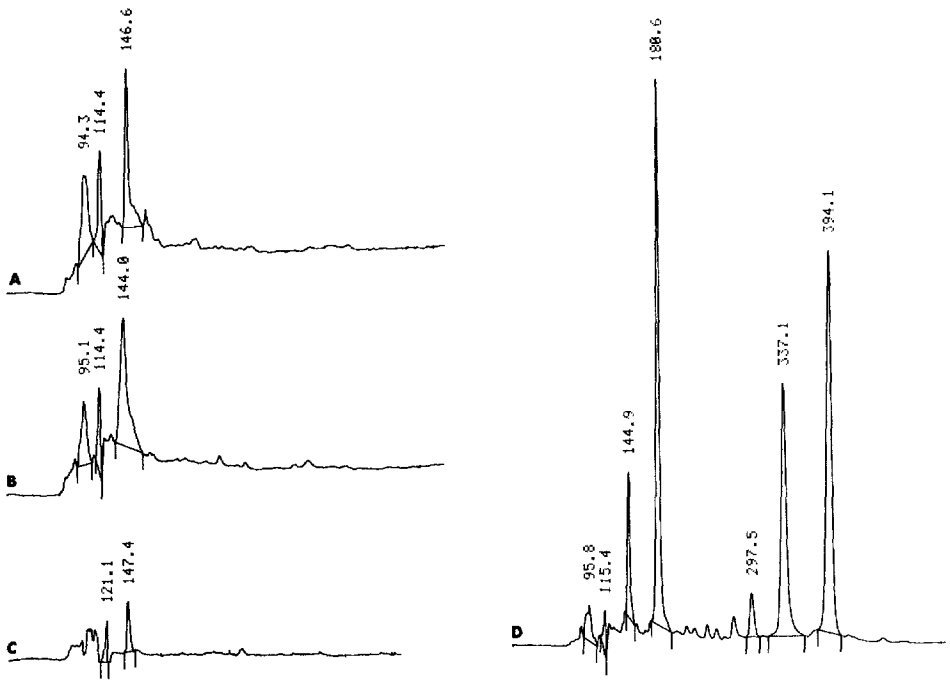

Fig. 6. Chromatograms (reductive detection) of "difficult" samples: *post mortem* bloods from non-benzodiazepine cases, putrefied (A, B) and undegraded (C); and a sample from a road traffic offence case that shows a strong peak (394.1 s) due to the particular brand of specimen container. Other peaks in D are internal standard (180.6 s), oxazepam (297.5 s) and temazepam (337.1 s). All the chromatograms are scaled to the same sensitivity. The peak amplitude of the internal standard peak (D) is 10.2 nA.

this position. The problems caused to some other analytical techniques by this type of interference are notorious[4,5].

Analytical considerations and results

For usual reasons it is desirable that in internally standardized determinations the standard should be a benzodiazepine closely matched to the adsorption characteristics of the analyte on both Porapak-T and octadecylsilyl silica, and to the analyte's electrochemical characteristics. In a drugs-abuse case, however, several benzodiazepines may be present. When a number of such samples are collectively in process, the choice of a benzodiazepine to occupy a position vacant in all of the chromatograms may not be possible. The 6-nitroquinoline used in the present work has proved to be a useful compromise, found after a considerable search, when no benzodiazepine is available, although some sacrifice in accuracy for some analytes is involved. A number of relevant characteristics of the compound are given in Tables I and III.

The results, standardized with the nitroquinoline, from a blind trial are given in Table IV. The person preparing the trial was asked to make up five representative samples with any of the benzodiazepines on the limited list, and their metabolites. There was no specification of how many compounds were to be used of the complete

TABLE IV

BLIND TRIAL ON SPIKED, HAEMOLYZED HUMAN BLOOD SAMPLES

$n = 3$.

Sample No.	*Compound*	*Spike (ng/ml)*	*Found*	
			Range (ng/ml)	*Mean error (%)*
1	7-Aminonitrazepam	199	206–252	+13.1
	Nitrazepam	99.6	101–103	+1.84
	Lorazepam	60.0	57.6–61.5	−0.89
2	Temazepam	99.0	87.6–89.4	−10.8
	Desmethyldiazepam	297	274–277	−7.42
	Diazepam	594	56.9–576	−3.49
3	Triazolam	99.6	93.2–98.2	−3.41
	Oxazepam	199	182–190	−6.51
	Temazepam	99.6	95.9–96.0	−3.68
4	Demoxepam	199	181–193	−5.35
	Chlordiazepoxide	99.6	89.9–95.0	−6.62
	Desmethyldiazepam	59.8	57.1–60.4	−1.39
5	Loprazolam	59.3	56.8–58.7	−3.04
	Lormetazepam	59.3	57.0–59.0	−1.91

set made available. As the table shows, all the compounds were correctly identified, with no false positives; and the quantitative results are within reasonable agreement, on the basis of the data already given, with the quantities added to the blood.

CONCLUSION

The described techniques have considerably increased the facility with which the benzodiazepines can be detected and determined in degraded samples, and have made possible a variety of novel observations that will be reported elsewhere. During the course of this work it has become apparent that the cleanup procedure might provide the basis of a more comprehensive scheme, and that a variety of other compounds of casework interest are amenable to reductive detection. We look forward to pursuing these points.

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