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RAPID MICRO-METHOD FOR THE MEASUREMENT OF DIAZEPAM AND DESMETHYLDIAZEPAM IN BLOOD PLASMA BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid gas–liquid chromatographic technique has been developed for use in the simultaneous measurement of both diazepam and its major metabolite, desmethyldiazepam, in blood plasma. Neither solvent transfer nor evaporation steps are required in this procedure. Extremely small plasma volumes (20 to 100 μl) are used and this has proved advantageous, especially when analysing samples from neonates. A linear response to amounts from $1 \cdot 10^{-12}$ to $1 \cdot 10^{-9}$ g/sec of both diazepam and desmethyldiazepam has been obtained and thus the direct analyses of these compounds both during therapy and after overdosage has been possible. Interference from either endogenous or exogenous sources has been found to be minimal.

INTRODUCTION

The simultaneous measurement of diazepam and its metabolites in biological fluids has been a subject of much investigation[1,2]. Very sensitive techniques are required in this analysis since drug concentrations encountered during diazepam therapy are usually less than 1 mg/l. Although a radioimmunoassay has been developed for the measurement of both diazepam and desmethyldiazepam[3], its use may be restricted not only by the low linear range available, but also by the cross reaction of diazepam with antibodies specific for desmethyldiazepam, and by the time (approximately 24 h) needed to complete the assay.

The technique used most extensively in the analysis of diazepam and its metabolites has been gas–liquid chromatography (GLC) with electron capture detection. The major disadvantages in the use of this technique have been the lengthy purification procedures and the large volumes (1 to 2 ml) of plasma required, but recently more rapid analytical procedures have been developed. Berlin *et al.*[4] described a method for the direct extraction of diazepam and desmethyldiazepam in which benzene was used as the solvent and griseofulvin as the internal standard. This procedure has been reproduced with several minor modifications[5,6], but both of these techniques contain potential sources of error. Arnold[5] used chlordiazepoxide as the internal standard, although this compound has been shown to be thermally labile

under similar chromatographic conditions[7]. The chromatography of Gamble *et al.*[6] revealed excessive tailing of both desmethyldiazepam and griseofulvin and, since drug concentrations were calculated from measurements of peak height ratios, such pronounced tailing would be expected to increase the possibility of analytical error. Although the technique described by Howard *et al.*[8] was simple and potentially rapid, not only were 2 ml of plasma required, but also emulsions were said to form irrespective of the mixing techniques used.

The method described here was based upon an analogous technique used for the direct extraction and analysis of barbiturates and some other hypnotics[9,10]. The principle of rapid solvent extraction followed by analysis of the extract without concentration is common to both procedures; any volume from 20 to 100 μl of plasma may be used, and, provided that an equal volume of solvent is added, neither the recovery of drug nor the sensitivity of the method are changed. A single Dreyer tube is used in the extraction and therefore problems of either cross-contamination or sample loss during solvent transfer are avoided. Concentrations of both diazepam and desmethyldiazepam from 0.02 to 25.0 mg/l can be measured without either concentration or dilution of the sample.

MATERIALS AND METHODS

Chemicals and reagents

The internal standard, prazepam, was supplied by Warner-Lambert (Eastleigh, Great Britain). Diazepam and desmethyldiazepam were provided by Roche Products (Welwyn Garden City, Great Britain).

Two internal standard solutions, containing 0.4 mg/l (solution A) and 4.0 mg/l (solution B) of prazepam, were prepared in the extraction solvent, *n*-butyl acetate (analytical-reagent grade; Fisons, Loughborough, Great Britain).

Gas–liquid chromatography

A Pye 104 Model 84 gas chromatograph equipped with a 10-mCi ^{63}Ni electron capture detector and a GCV pulse-frequency-modulated amplifier was used. The chromatograph was modified so that all the column effluent passed to the above detector. The instrument conditions used in this assay were: carrier gas (argon) flow-rate, 80 ml/min; column oven temperature, 250°; detector oven temperature, 270°. The detector current setting was one half of the runaway current.

The column, a coiled glass tube 2 m × 4 mm I.D., was treated for 4 h with a solution of 2% dimethyldichlorosilane in toluene. After having been washed with methanol and then dried at 100°, the column was packed with 3% OV-7 on 80–100 mesh Varaport 30. The column packing was prepared using the rotating evaporator technique. The packed column was conditioned at 300° for 60 h with an argon flow-rate of 60 ml/min. The column was subsequently deactivated by means of frequent 3-μl injections of a 5 g/l solution of dipalmitoyl phosphatidylcholine (DPPC) (Sigma, Kingston-upon-Thames, Great Britain) in ethanol. This treatment decreased the retention time of desmethyldiazepam by approximately 10% and the broad, tailing peak initially obtained became sharp and almost symmetrical. To maintain the column in this deactivated state and thus to achieve the reproducible chromatography of desmethyldiazepam, 3 μl of the DPPC solution were injected every 4 h while the

column was in use. Treatment of the column in this manner had no effect on the chromatography of either diazepam or prazepam. Similar effects of other phospholipids on the behaviour of silicone columns have been observed (Leitch *et al.*[11]; C. Hallett, personal communication).

The retention times of diazepam, its metabolites in man (desmethyldiazepam, oxazepam and temazepam) and prazepam on this system are given in Table I. The chromatography of diazepam, desmethyldiazepam and prazepam is illustrated in Fig. 1.

TABLE I

THE RETENTION TIMES OF DIAZEPAM, ITS METABOLITES IN MAN AND SOME OTHER BENZODIAZEPINES ON THE 3% OV-7 COLUMN SYSTEM

The amount of each compound injected on to the column was 2 ng.

Compound	*Retention time (min)*	*Retention time (relative to prazepam)*
Medazepam	2.0	0.34
Oxazepam*	2.7	0.45
Lorazepam*	3.3	0.55
Diazepam	3.6	0.59
Desalkylflurazepam	4.1	0.68
Desmethyldiazepam**	4.7	0.78
Demoxepam*,**	4.7	0.78
Prazepam	6.0	1.00
Hydroxyethylflurazepam**	6.9	1.15
Temazepam*	7.3	1.21
Flurazepam	7.9	1.31
Nitrazepam**	10.6	1.76
Chlordiazepoxide*	12.1	2.01
Clonazepam**	12.7	2.12

* These compounds have been found to be unstable under similar GLC conditions[7,13].

** These compounds gave tailing peaks on this system.

Extraction procedure

A 100-μl volume of plasma was introduced into a Dreyer tube (Poulten, Selfe and Lee, Wickford, Great Britain) by means of a 100-μl semi-automatic pipette. Then 100 μl of the appropriate internal standard solution were added to the tube via a 5.0-ml Hamilton gas-tight glass syringe, fitted with a Hamilton repeating mechanism (obtained from Field Instruments, Richmond, Great Britain). Internal standard solution A was used in the measurement of drug concentrations below approximately 1 mg/l. However, if the concentration of drug in the sample was expected to exceed this value, solution B was used. The contents of the tube were mixed thoroughly on a Vortex mixer for 30 sec and centrifuged at approximately 1700 *g* for 5 min. Without disturbing the lower (aqueous) phase, 2 μl of the *n*-butyl acetate extract were carefully withdrawn into a 5-μl SGE syringe fitted with an 11.5-cm needle and injected onto the column of the gas chromatograph. All plasma samples were analysed in duplicate.

Quantitation

Two ranges of standard solutions were prepared in *n*-butyl acetate. The first

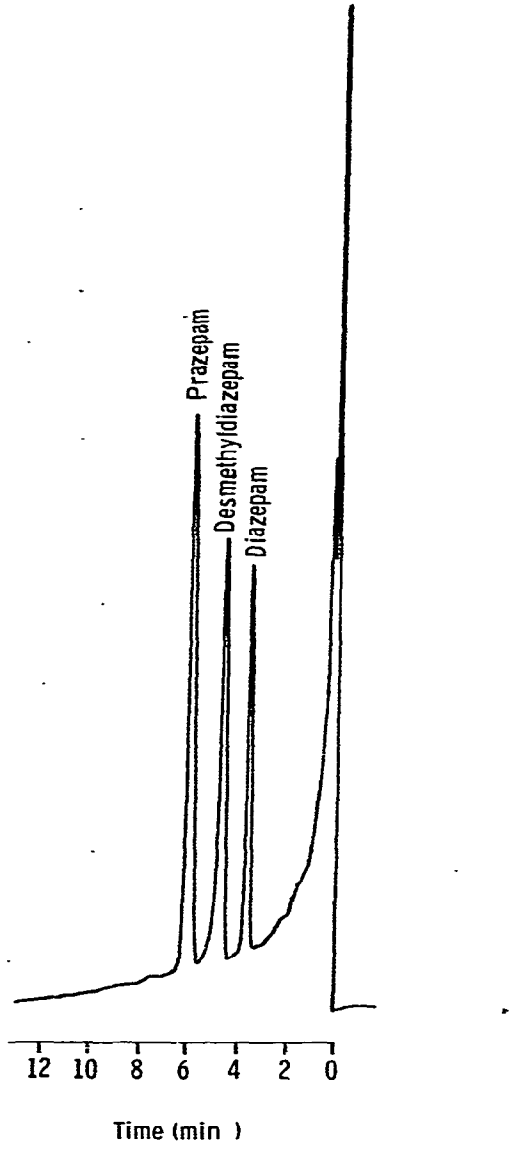


Fig. 1. The chromatography of an *n*-butyl acetate solution containing 0.2 mg/l of both diazepam and desmethyldiazepam; the concentration of prazepam was 0.4 mg/l. Volume injected: 2 μl; amplifier attenuation: $\frac{1}{2}\times$ 64.

contained from 0.05 to 1.0 mg/l of diazepam and desmethyldiazepam and 0.4 mg/l of prazepam while the second contained from 1.0 to 10.0 mg/l of diazepam and desmethyldiazepam and 4.0 mg/l of prazepam. All standard solutions were prepared by dilution from separate stock solutions containing 100 mg/l of each compound in *n*-butyl acetate. These solutions were found to be stable for at least three months when stored at 4° and in the absence of light.

The ratio of the peak height of drug to the peak height of prazepam, when plotted against drug concentration, was linear for both diazepam and desmethyldiazepam over the above concentration ranges. The use of the appropriate concentration of prazepam in both sample extracts and standard solutions was essential in the measurement of this wide range of drug concentrations; measureable detector responses to the three benzodiazepines could thus always be achieved without alteration of the attenuation of the detector response during the chromatographic analysis. Typical calibration gradients obtained using a prazepam concentration of 4 mg/l were 0.35 l/mg and 0.39 l/mg for diazepam and desmethyldiazepam, respectively; for the solutions containing 0.4 mg/l of prazepam, these respective values were 3.5 l/mg and 3.9 l/mg.

RESULTS AND DISCUSSION

Recovery studies

Solutions containing both diazepam and desmethyldiazepam at concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 4.0, and 8.0 mg/l were prepared in heparinised bovine plasma. Each solution was analysed in quadruplicate with the use of the appropriate internal standard solution. The mean percentage recoveries for diazepam and desmethyldiazepam, calculated from a total of 36 analyses, were 100 (S.D. = 4) and 100 (S.D. = 5), respectively.

Specificity

Endogenous compounds. No endogenous components of plasma have been found to interfere in this assay. A typical chromatogram obtained following the *n*-butyl acetate extraction of drug-free heparinised human plasma is shown in Fig. 2. The "negative" peak with a retention time of 13.4 min (2.23 relative to prazepam) was observed in the chromatograms of most plasma extracts; it did not interfere with any of the drugs under study. The compound responsible for this abnormal detector response has not been identified.

Non-benzodiazepine drugs. Interference from other drugs was rarely encountered, although chlorpromazine could give rise to interference since this compound had a retention time relative to prazepam of 0.62 on this system and therefore eluted in close proximity to diazepam. However, chlorpromazine has an electron capture response which is approximately one quarter of that of diazepam and, since plasma concentrations of chlorpromazine during chronic therapy seldom exceed 0.3 mg/l (ref. 12), any interference from this source is unlikely to be significant. Using the present method, chlorpromazine has not been detected in any plasma samples obtained from patients receiving doses of this drug from 100 to 1000 mg daily. Nevertheless, if an overdose of chlorpromazine were to be taken in combination with diazepam, some interference could arise. In this situation it would be necessary to resort to a second GLC column based upon an alternative stationary phase (*e.g.* OV-1 or OV-225) to resolve these compounds.

Benzodiazepines. Desmethyldiazepam is a metabolite not only of diazepam, but also of three other commonly prescribed benzodiazepines: medazepam, chlordiazepoxide and dipotassium chlorazepate. Thus, detection of this metabolite in plasma cannot be regarded as evidence of the ingestion of diazepam unless it can be

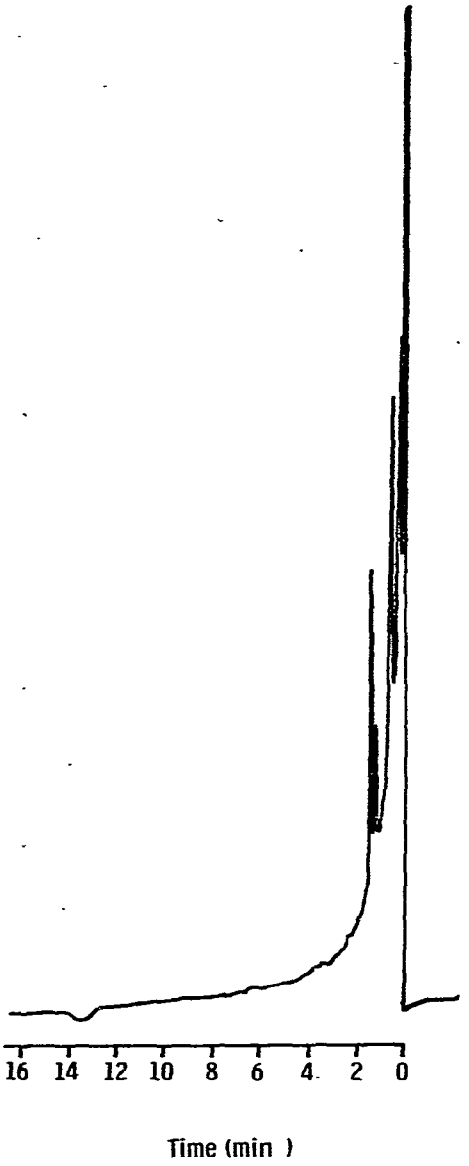


Fig. 2. The chromatography of an *n*-butyl acetate extract of drug-free plasma without the addition of internal standard. Volume of extract injected: 2 μl; amplifier attenuation: × 64.

established that diazepam was the only one of the above benzodiazepines taken. A further problem may arise if chlordiazepoxide has been ingested. An additional metabolite of this drug, demoxepam (*i.e.* the N-oxide analogue of desmethyldiazepam), gave rise to a peak which had the same retention as desmethyldiazepam on this chromatographic system (Table I). Since N-oxides such as demoxepam are thermally labile[7], the peak observed on the chromatogram after an injection of this drug is most likely to be its major thermal decomposition product, *i.e.* desmethyldiazepam.

It should be noted that diazepam also is a metabolite of medazepam, and thus the presence of diazepam in a plasma sample does not, by itself, preclude the possibility that medazepam was ingested.

No other benzodiazepines available in the United Kingdom have been found to interfere in this assay. The retention times of these drugs and some of their metabolites are shown in Table I.

The hydroxy metabolites of diazepam (oxazepam and temazepam) were infrequently detected in plasma samples from patients receiving chronic diazepam therapy. Fig. 3 shows the presence of temazepam in plasma obtained from a patient who had been treated with 15 mg/day of diazepam for 7 years. However, after the ingestion of excessive doses of diazepam, these metabolites could usually be detected, as illustrated in Fig. 4. In nearly all of these cases, the concentrations of both oxazepam and temazepam were considerably lower than those of either diazepam or desmethyldiazepam.

Although oxazepam and temazepam may be readily identified when present in plasma samples, difficulties have been experienced in the quantitative analyses of these drugs since the chromatography of each metabolite on this system has been found to be variable. It is possible that this non-reproducible behaviour is a consequence of the partial thermal decomposition of these hydroxy compounds[7,13].

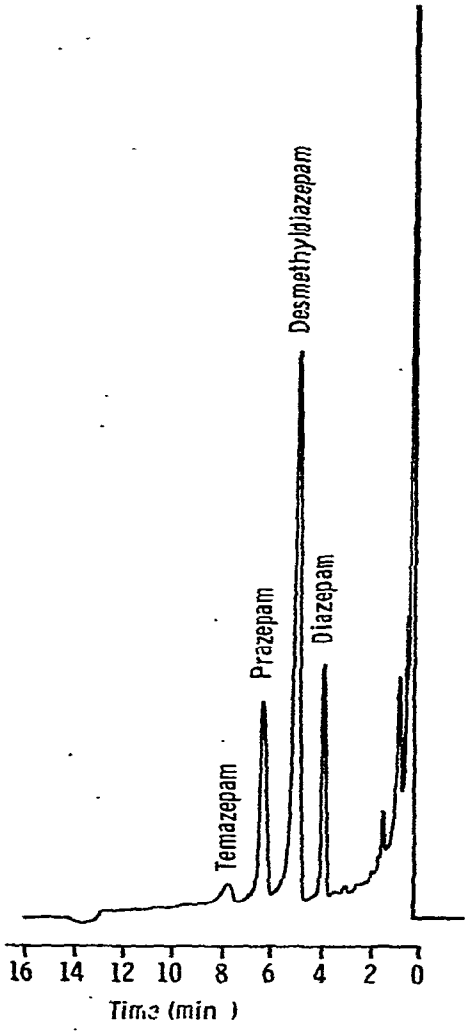
Choice of internal standard. Prazepam was found to be the most suitable compound to use as an internal standard in this analytical procedure. Its advantages were: (i) it was a benzodiazepine with structural similarities to the drugs under study, (ii) it was not marketed in the United Kingdom, (iii) when chromatographed on the OV-7 column system, it gave rise to a sharp, symmetrical peak that was well resolved not only from both diazepam and desmethyldiazepam, but also from any other possible sources of interference, and (iv) it was stable in *n*-butyl acetate solution.

Extraction conditions

Solvent. The extraction solvent, *n*-butyl acetate, was selected for the following reasons: (i) it is almost immiscible with aqueous solutions, (ii) it has a poor electron capture response, (iii) most benzodiazepines are very soluble in this solvent and (iv) emulsions of the solvent and plasma were rarely encountered; however, if they did occur, it was found that centrifugation for a further 5 min was usually sufficient to yield a clear extract.

Extraction time. The influence of the extraction time on the recoveries of both diazepam and desmethyldiazepam from plasma was investigated. Extractions were performed for 15, 30, 45 and 60 sec, respectively. No significant variations in the recoveries of either compound occurred if mixing was continued for 30 sec or longer. Similar mean recoveries were also obtained after mixing for 15 sec, although the standard deviations of these values were greater than those obtained when longer mixing times were used. Therefore 30 sec was chosen as the optimum extraction time. The mixing time had no apparent effect on the amount of co-extracted endogenous material present in the extract.

pH. Since the pH of human plasma is normally between 7.3 and 7.5 (ref. 14), the addition to plasma of a buffer of similar pH would not be expected to improve the efficiency of benzodiazepine extraction. To confirm this, 100 μl of an aqueous disodium hydrogen orthophosphate solution (1 mole/l; pH 7.4) were added to 100 μl



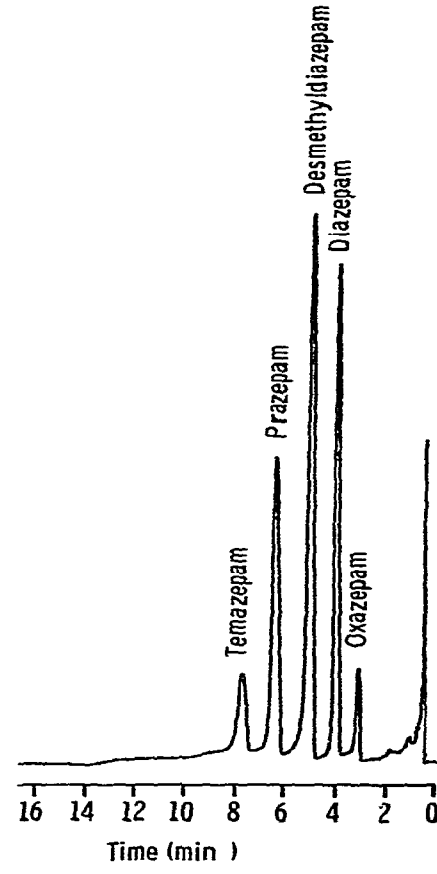


Fig. 3. The chromatography of an extract of plasma from a patient receiving 15 mg of diazepam daily. The concentrations of diazepam and desmethyldiazepam were 0.35 and 0.76 mg/l, respectively; the concentration of prazepam was 0.4 mg/l. Volume of extract injected: 2 μl; amplifier attenuation: × 128.

Fig. 4. The chromatography of an extract of plasma from a patient who had ingested an overdose of diazepam. Both the dose and time interval between dose and blood sampling were unknown. The concentration of diazepam was 4.75 mg/l and that of desmethyldiazepam was 4.61 mg/l; the prazepam concentration was 4 mg/l. Volume of extract injected: 1 μl; amplifier attenuation: × 512.

of heparinised bovine plasma containing both diazepam and desmethyldiazepam at concentrations of 0.4 mg/l. The subsequent analyses of this mixture, both with and without the addition of buffer, revealed that the recoveries of these drugs were identical. For this reason, a buffer solution was not included in the extraction procedure. Similarly, when benzene was used as the extraction solvent, it was found[5] that the addition of an appropriate buffer to maintain pH values at either 7 or 8 had no significant effect on drug recoveries.

However, if the pH of the plasma specimen for analysis is likely to greatly exceed the normal limits (*e.g.* in some forensic samples), the addition of a pH 7.4 buffer may be necessary to achieve the recoveries reported here.

Sensitivity

Since both diazepam and desmethyldiazepam have very large electron capture responses as little as $1 \cdot 10^{-12}$ g/sec (*i.e.* 20 pg) of each compound can be detected on column. When operating the ^{63}Ni detector in the pulsed mode, with pulse intervals of either 150 or 500 μsec, the linearity of the response to both compounds was restricted severely. Thus, all plasma samples containing concentrations above 0.8 mg/l of either drug had to be diluted before analysis. Not only was this procedure time-consuming, but also the reproducibility of the measurement was reduced. By the use of a pulse-frequency-modulated amplifier[15], a linear response to amounts from $1 \cdot 10^{-12}$ to $1 \cdot 10^{-9}$ g/sec of each compound was achieved and thus the need to dilute samples was obviated.

With a plasma to solvent ratio of 1:1 in the extraction procedure, concentrations of diazepam and its desmethyl metabolite down to 0.02 mg/l could be measured in plasma, without either prior concentration or purification of the extract. However, it has been found that the sensitivity of the technique may, if necessary, be enhanced. The plasma to solvent ratio can be increased up to 4:1 without affecting the recovery of either drug, and no increase in the frequency of emulsion formation has been observed during the analysis of freshly obtained plasma specimens. Similarly, no endogenous materials have been found to interfere in this modified analytical procedure. Thus, with this simple extraction method it has proved possible to measure plasma concentrations of diazepam and desmethyldiazepam down to 0.005 mg/l.

In cases where the quantity of specimen available was restricted, *e.g.* heel prick samples from neonates, the extraction was performed using 20 μl of both the plasma and the internal standard solution. Thus, the plasma to solvent ratio remained 1:1 and a correction for the change in extract volume was unnecessary. Finely tapered glass tubes approximately 5 mm or less in diameter (J. Service, Beckenham, Great Britain) were used in the extraction of these small sample volumes. Dreyer tubes, which are slightly larger in diameter and more rounded at the bottom, were found to be unsuitable for such volumes.

Practical application of the technique

The analytical procedure has been in routine use in this laboratory for over two years. During that time, it has been applied successfully to the analysis of diazepam and desmethyldiazepam in plasma samples obtained from patients in a variety of clinical conditions. These have included: neonates whose mothers had received diazepam during pregnancy for the treatment of pre-eclamptic toxaemia, patients of all ages who had been treated with this drug for the control of seizures, and adults who had received diazepam in the treatment of chronic anxiety. Plasma samples derived from patients who had ingested an overdose of diazepam were also conveniently analysed by use of this method. A wide range of drug concentrations (from 0.02 to 25 mg/l) has been encountered in these specimens.

Conclusion

The method described in this paper for use in the analysis of diazepam and its major metabolite, desmethyldiazepam, in plasma offers advantages in both the analysis time and the amount of sample required. A complete quantitative analysis can be performed, in duplicate, within 30 min. The extraction is carried out in a single tube and no solvent evaporation procedure is necessary. Thus, the problems of drug loss which can occur during both solvent transfer and evaporation stages are eliminated. The technique has proved to be extremely flexible, enabling extractions to be performed on as little as 20 μl of plasma with no loss of sensitivity. The limit of sensitivity was normally 0.02 mg/l for both diazepam and desmethyldiazepam, but when the plasma to extraction solvent ratio was increased to 4:1, this limit was correspondingly increased to 0.005 mg/l. The selectivity of the electron capture detector, in combination with both the column system based upon the silicone liquid phase OV-7 and the solvent extraction procedure, has ensured that problems of interference in the assay are few.

The potential applications of this technique in the qualitative and quantitative analysis of other benzodiazepines, especially in the field of clinical toxicology, are currently under investigation. Preliminary results indicate that most of the benzodiazepines currently prescribed in the United Kingdom can be resolved and measured by use of this technique.

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