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Note

Use of internal standards in the estimation of diazepam in plasma

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Prior to a clinical study of the relationship between diazepam concentrations in plasma, within minutes of its administration, and other factors[1], a method for its estimation in plasma, incurring minimal error, was sought.

The estimation of drug concentrations in biological fluids is sometimes based on figures for the recovery of the drug on extraction of standard amounts[2–4]. However, this method does not account for variation in recovery from sample to sample. The latter variation is considered to be allowed for by the use of internal standards

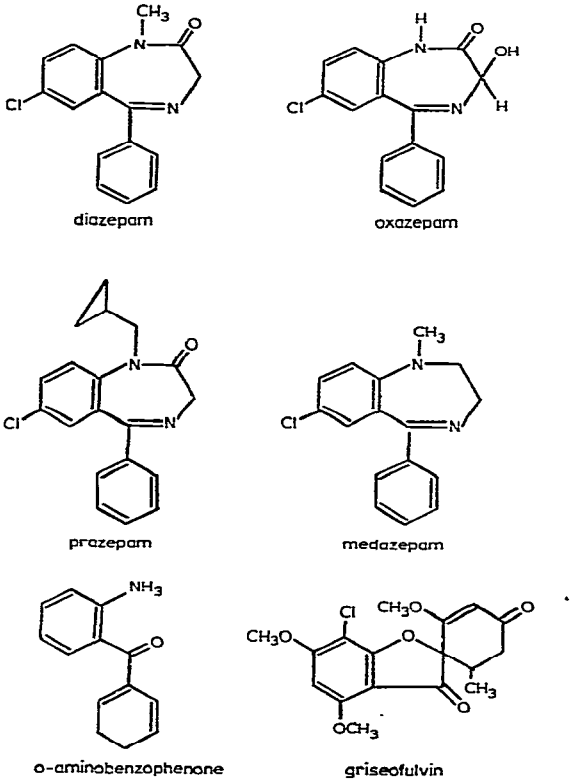

Fig. 1. Structural formulae of diazepam and internal standards.

in extraction. Thus, in the estimation of plasma diazepam, griseofulvin (see Fig. 1) has been used for extraction into benzene[5], prazepam for extraction into ethyl benzoate[6], medazepam for extraction into ether, thence to 2 mol/l HCl and back into ether[7] and O-aminobenzophenone for extraction into ether, into 6 mol/l HCl in which hydrolysis to 2-methylamino-5-chlorobenzophenone was carried out and the hydrolysate then extracted into ether[8]. It is assumed[7] that high recoveries of drug and internal standard, which are of comparable magnitude, indicate reliability of the internal standard as such. It was decided to check this assumption.

METHODS

Oxazepam (N-desmethyl-3-hydroxydiazepam) and prazepam (7-chloro-1-cyclopropylmethyl-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one) were to be tried as internal standards. On the basis of previous reports, it was not expected to find the metabolites of diazepam, N-desmethyldiazepam[9] and oxazepam[4,10–12] in blood samples for the proposed clinical study. These would have interfered with the chromatographic analysis of the internal standards.

Fifteen samples of plasma to which oxazepam and diazepam had been added and eleven to which prazepam and diazepam had been added were analysed.

Extraction

The extraction was based on the data of Zingales[4]. Various amounts of diazepam and internal standard were dissolved in 1-ml samples of plasma which, upon buffering to pH 9.5 and saturation with $(NH_4)_2SO_4$ were extracted with 5.0 ml toluene–isoamyl alcohol (96:4). Extraction into 1.0 ml 6 mol/l HCl followed. After washing with diethyl ether and neutralization in ice with 6 mol/l NaOH, extraction into 5.0 ml toluene–isoamyl alcohol was carried out. The latter phase was rotary evaporated at 47° under a vacuum of 125 Torr, the residue quantitatively collected as previously described[13] and dissolved in 100 μl toluene–isoamyl alcohol of which 2-μl injections were used for chromatographic analysis.

Chromatography

A Pye GCV gas chromatograph and ^{63}Ni pulse-modulated electron-capture detector were used with 1.5 m×4 mm I.D. borosilicate glass columns packed with 3% OV-17 on 60–80 mesh Gas-Chrom Q. Carrier gas: argon, flow-rate 85 ml/min. Temperatures: injector zone, 265°, column, 245°, detector, 330°. Under these conditions, diazepam elutes with a retention time of approximately 4 min and, relative to this, oxazepam and prazepam have retention times of 0.7 and 1.7, respectively.

RESULTS

Data are presented in Tables I and II. It can be seen that there is greater variation in the recovery of oxazepam relative to diazepam than in that of prazepam and while diazepam and oxazepam recoveries are apparently unrelated ($r = 0.19$) (see Fig. 2), those of prazepam and diazepam (see Fig. 3) are highly correlated: prazepam recovery (%) = 0.87 diazepam recovery (%) + 1.07 ($r = 0.84$, $P < 0.01$). The mean ratio of diazepam recovery to internal standard recovery was 1.232 for

TABLE I

RECOVERIES OF CO-EXTRACTED DIAZEPAM AND OXAZEPAM

Amount of oxazepam extracted (μg)	*Amount of diazepam extracted (μg)*	*Oxazepam recovered, % (A)*	*Diazepam recovered, % (B)*	*B/A*
8.24	9.94	81.6	99.4	1.218
8.24	9.94	64.8	91.0	1.404
8.24	9.94	69.7	99.3	1.425
145.6	153.0	69.0	100.1	1.451
145.6	153.0	83.2	101.7	1.222
145.6	153.0	61.2	95.8	1.565
8.72	9.06	82.4	111.9	1.358
8.72	9.06	78.7	114.2	1.451
8.72	9.06	85.8	119.5	1.393
8.72	9.06	103.3	99.9	0.967
8.72	9.06	107.0	106.5	0.995
0.489	0.325	96.4	103.2	1.071
0.489	0.325	99.8	104.8	1.050
0.489	0.325	104.7	98.7	0.943
0.489	0.325	100.0	96.2	0.962
	mean	85.8	102.8	1.232
	S.D.	15.346	7.552	0.2171
	1.96 S.D.	30.078	14.802	0.425
	S.D./mean	0.179	0.074	0.176
	S.E.	3.96	1.95	0.065

TABLE II

RECOVERIES OF CO-EXTRACTED DIAZEPAM AND PRAZEPAM

Amount of prazepam extracted (μg)	*Amount of diazepam extracted (μg)*	*Prazepam recovered, % (Q)*	*Diazepam recovered, % (R)*	*R/Q*
0.564	0.512	82.8	98.5	1.190
2.256	2.048	84.9	93.7	1.104
3.948	3.582	86.7	95.9	1.106
5.639	5.118	88.8	99.2	1.117
0.556	0.443	70.3	82.5	1.173
2.222	1.77	78.7	90.9	1.155
3.889	3.099	84.9	98.4	1.159
5.556	4.428	81.8	97.5	1.192
0.0518	0.0387	81.8	93.7	1.145
0.0907	0.0677	82.5	89.9	1.090
0.0295	0.0968	88.6	96.2	1.086
	mean	82.9	94.2	1.138
	S.D.	5.17	4.97	0.039
	1.96 S.D.	10.13	9.74	0.076
	S.D./mean	0.062	0.053	0.034
	S.E.	1.56	1.5	0.012

oxazepam and 1.138 for prazepam. Thus, using oxazepam as internal standard, the 95% limits for the ratio in plasma of diazepam/oxazepam concentrations are given by 1/(1.232 ± 0.425) times diazepam/oxazepam concentrations in extracts, *i.e.* within 74.3% and 152.7% of 1/1.232 diazepam/oxazepam concentrations in extracts. When prazepam is used as internal standard, the corresponding 95% limits are given by 1/(1.138 ± 0.076) times the ratio diazepam/prazepam concentrations in extracts or within 93.75% and 107.15% of 1/1.138 times this ratio.

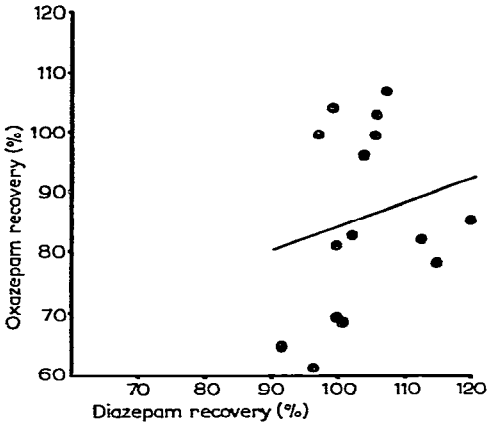

Fig. 2. Relationship between oxazepam and diazepam recoveries.

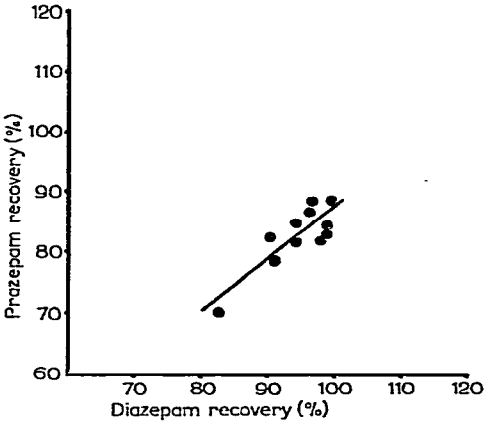

Fig. 3. Relationship between prazepam and diazepam recoveries.

DISCUSSION

Owing to the lack of a relationship between oxazepam and diazepam recoveries, the use of oxazepam as internal standard can lead to an error in the estimation of plasma diazepam of up to 50%. Prazepam, on the other hand, on account of its parallel recovery with diazepam, can be used to find plasma diazepam concentration to within 7%.

In this study, drug and internal standard were carried through the same extraction procedures. The difference in extraction behaviour of oxazepam and prazepam relative to diazepam is, no doubt, a result of the greater structural and chemical likeness of prazepam to diazepam than of oxazepam to diazepam.

The need for caution in the use of internal standards is all the more necessary when drug and internal standard are not subjected to exactly the same procedures.

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