Journal of Chromatography, 494 (1989) 257–265
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4871

DETERMINATION OF AN OXADIAZOLE-SUBSTITUTED 1,4-BENZODIAZEPINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION AND BY A RADIORECEPTOR ASSAY

LARS NORDHOLM* and HELLE MENGEL

Departments of Drug Metabolism and Pharmacokinetics, Ferrosan Research Division, Sydmarken 5, DK-2860 Soeborg (Denmark)

(First received January 16th, 1989, revised manuscript received May 12th, 1989)

SUMMARY

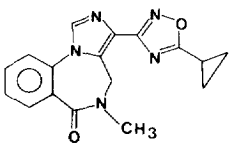
A high-performance liquid chromatographic (HPLC) method for the determination of an oxadiazole-substituted 1,4-benzodiazepine [3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5a][1,4]benzodiazepine] in plasma has been developed and compared with a radioreceptor assay The results given by the two methods were in good agreement, with detection limits of ca 1 ng/ml (signal-to-noise ratio = 3) The radioreceptor method is preferred for the monitoring of toxicological and other well controlled studies, while HPLC is preferred where greater specificity is essential Further, the HPLC assay is applicable over a much wider concentration range

INTRODUCTION

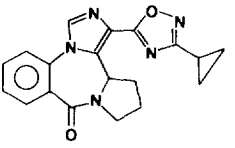
During the development of a new drug, several assays may be developed to acquire data with sufficient precision for specific purposes. In initial toxicological studies, it may be of greater importance to know the plasma levels as soon as the study is completed rather than to wait until the final assay has been completely validated At the latter point, however, it is of great importance to compare the initial and final methods.

In this paper we describe two assays for the determination of 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5a][1,4]-benzodiazepine (I) (Fig. 1) in plasma. Compound I is a potent benzo-

0378-4347/89/$03 50



I FG 8119



II FG 8123

Fig 1 Structures of compound I and the internal standard II

diazepine with a better separation between anxiolytic effects and side-effects than the current benzodiazepines. Being a ligand for the benzodiazepine receptor, compound I can be measured using a benzodiazepine radioreceptor assay [1] This assay has been used for monitoring plasma levels during the first toxicological studies in rats and monkeys

In parallel, a reversed-phase high-performance liquid chromatographic (HPLC) assay utilizing UV detection has been developed A number of chromatographic methods for the determination of other imidazobenzodiazepines in biological fluids have been developed previously, including HPLC with UV detection [2–6] and gas chromatography (GC) with electron-capture detection [7–10], nitrogen-sensitive detection [11] and negative ion chemical ionization mass spectrometry [12] All these methods have employed liquid–liquid extraction before chromatography

Compound I is a very potent substance and consequently a sensitive analytical method is required The UV maximum of the compound is 244 nm. Initial studies at this wavelength have shown that high sensitivity and reasonable selectivity can be obtained with human plasma samples when appropriate extraction procedures (e g , solid-phase extraction) are used In case of rat plasma and sometimes also monkey plasma, interfering endogenous substances may impair the selectivity.

EXPERIMENTAL

HPLC method

Chemicals Compound I and the internal standard 1-(3-cyclopropyl-1,2,4-oxadiazol-5-yl)-11,12,13,13a-tetrahydro-9-oxo-9*H*-imidazo[1,5-a]pyrrolo-[2,1-c][1,4]benzodiazepine (II) were synthesized at Ferrosan (Soeborg, Denmark). Water was obtained from a Milli-Q water purification system (Millipore, Molsheim, France) All organic solvents were of HPLC grade and all other chemicals were of analytical reagent grade Drug-free plasma samples used for validation of the method were made by use of heparin and obtained from a number of healthy human subjects taking no medication

Apparatus A Vac-Elut® SPS24 solid-phase extraction apparatus (Analytichem, Harbor City, CA, U S A) was used in the extraction step A Merck (Virum, Denmark) HPLC system consisting of an L 6200 pump, a 655A-40 au-

tosampler, an L4200 UV detector (244 nm) and a D2000 Chromato Integrator with a diskette station were employed. The column was 25 cm × 4 mm I D LiChrospher RP-18 end-capped, 5-μm particle size (Merck) The mobile phase was 30% (v/v) acetonitrile in 0.013 *M* sodium phosphate buffer (pH 7 0) at a flow-rate of 1 3 ml/min Analyses were carried out at 55 °C in order to reduce the back-pressure on the analytical column

Extraction procedure A Bond-Elut® C_{18} extraction column (200 mg, 3 ml) placed in the Vac-Elut unit (waste position) was washed twice with 1 ml of methanol and twice with 1 ml of water A 100-μl volume of 0.1 *M* sodium carbonate, 1.0 ml of plasma and 100 μl of a 1 μg/ml solution of internal standard II were added to the column, followed by gentle suction Two 1-ml volumes of water and 100 μl of methanol were then added successively with gentle suction in between The Vac-Elut unit was turned to the "collect" position and 100 + 200 + 200 μl of methanol were added with gentle suction in between The apparatus was allowed to stand with the suction on until the collected extracts were evaporated to approximately 50 μl The extracts plus 100 μl of water were mixed and transferred into autosampler microvials. A 100-μl volume was injected into the HPLC system Addition of water was used in order to improve the peak shapes in the chromatograms

Spiking of plasma samples was done either by addition of aqueous standards of compound I to plasma on the Bond-Elut column or by spiking of plasma pools (for analysis of samples larger than 1 ml).

Benzodiazepine radioreceptor assay (RRA)

The RRA has been described in detail previously [1]. In summary, plasma samples were extracted with ethyl acetate and after isolation and evaporation of the organic phase, the residue was dissolved in phosphate buffer and receptor suspension and [^{3}H]flunitrazepam were added After standing for 20–30 min, 10 ml of buffer were added, the samples were filtered through glass-fibre filters and the radioactivity on the filters was measured by liquid scintillation counting The concentration of compound I was calculated by comparison with standards

The only modification to the original method was in the redissolution step, where 100 μl instead of 50 μl of phosphate buffer were used in order to improve the recovery and reproducibility. All samples were assayed in duplicate.

RESULTS

HPLC method

Linearity and detection limits The method was found to be linear at levels up to 500 ng/ml in human plasma and 10 μg/ml in monkey plasma The absolute limit of detection using the standard procedure and a signal-to-noise ratio of 3 was approximately 2 ng/ml. If available, it was possible to use up to

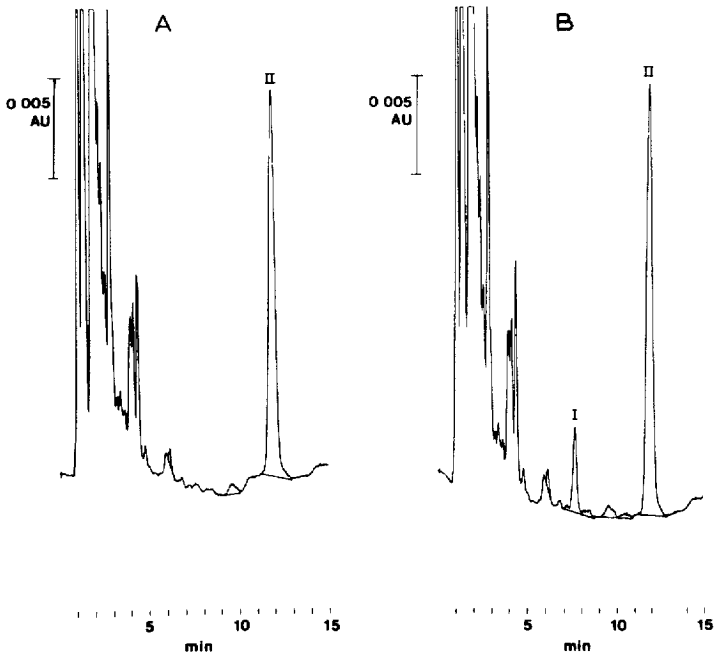


Fig 2 Chromatograms of (A) drug-free human plasma and (B) plasma spiked with 10 ng/ml of compound I

5 ml of plasma for the analysis In this instance the absolute limit of detection was approximately 0 5 ng/ml Using 5 ml of unspiked, drug-free plasma, analytical interference with the internal standard peak was observed in some instances

The method detection limit [13] was calculated as $t(n-1, 1-\alpha=0\ 95)S_m$, where $t(n-1, 1-\alpha=0\ 95)$ is Student's t-value for a one-tailed test at the 95% confidence level with $n-1$ degrees of freedom, and S_m is the standard deviation obtained on n replicates at the lowest concentration level in the intra-assay precision study Using these guidelines, the detection limit of the method was calculated to be 3 ng/ml This concentration is considered as the lowest reliable determination level

A chromatogram originating from a spiked plasma sample is shown in Fig 2

Recovery and precision The analytical recovery and intra-assay precision are given in Table I

The absolute recovery of I is 65%, determined by analysing ^{14}C-labelled I in the concentration range 3–13 ng/ml in plasma employing liquid scintillation counting

Inter-assay precision was measured by processing samples from the same plasma pools four times during a period of 2 months No sign of instability of I was observed The results are given in Table II.

TABLE I

RECOVERY, INTRA-ASSAY ACCURACY AND PRECISION OF THE DETERMINATION OF COMPOUND I IN PLASMA BY HPLC WITH UV DETECTION

Nominal concentration (ng/ml)	Concentration found (mean ± S D , $n=6$) (ng/ml)	Accuracy (% found)	Precision (C V , %)
5 0	5 28 ± 1 11	106	21
10 0	10 12 ± 0 27	101	2 7
20 0	20 45 ± 1 01	102	4 9
50 0	49 72 ± 1 63 ($n=5$)	99	3 3
300 0	308 08 ± 14 5	102	4 7

TABLE II

INTER-ASSAY ACCURACY AND PRECISION OF THE DETERMINATION OF COMPOUND I IN PLASMA BY HPLC WITH UV DETECTION

Concentration added (ng/ml)	Concentration found (mean ± S D , $n=4$) (ng/ml)	Accuracy (% found)	Precision (C V , %)
5	4 60 ± 0 39	92	8 5
10	10 13 ± 0 46	101	4 5
25	24 10 ± 1 02	96	4 2

Specificity The method was applied to rat and monkey plasma in addition to human plasma. Owing to many interfering peaks in the chromatogram in samples from these two species (especially the rat), it is not possible to monitor at the low nanogram level with the described method

Human plasma from a number of healthy subjects was assayed In some instances small interferences in the chromatogram (corresponding to less than 0 5 ng I per ml) were found

A few other drugs were investigated in order to evaluate possible interferences, including paracetamol, diazepam, lorazepam, carbamazepine and caffeine No interference was found except for diazepam, which, owing to a long retention time may interfere in subsequent chromatograms On-going studies of the metabolism of I indicate that all metabolites in rats and monkeys are considerably more polar than I and will consequently not interfere with the assay

RRA method

Linearity and detection limit The graph of the response (degree of binding) as a function of amount of substance in a given sample is sigmoidal Consequently, the graph can only be considered linear in the range corresponding to

TABLE III

RECOVERY OF ^{14}C-LABELLED I IN PLASMA SPIKED IN DUPLICATE AND EACH ASSAYED TWICE BY RRA

Step	Recovery (mean ± S D, $n=4$) (%)	
	2 5 ng/ml (ca 150 dpm per sample)	20 ng ml (ca 800 dpm per sample)
Evaporation	96 ± 3 6	95 ± 2 2
	–	92 ± 1 3
	–	98 ± 0 6
Redissolution in buffer	87 ± 1 8	88 ± 1 9
	–	91 ± 0 6
	–	93 ± 0 6

TABLE IV

INTRA-ASSAY PRECISION ($n=6$) IN PLASMA SPIKED WITH COMPOUND I ON FIVE DIFFERENT DAYS (RRA)

Approximate concentration (ng/ml)	C V (%)	Approximate concentration (ng/ml)	C V (%)
2 5	3	10	3
	12		1
	18		7
	5		5
	6		7
5	2	20	4
	12		1
	5		10 ($n=5$)
	6		7
	5		11

TABLE V

INTER-ASSAY PRECISION IN PLASMA SPIKED WITH COMPOUND I (ASSAYED OVER SIX WEEKS) (RRA)

Nominal concentration (ng/ml)	n	Mean concentration found (ng/ml)	C V (%)
2 5	11	2 4	12
5	11	4 3	9
5	9	4 5	10
10	11	8 5	14
10	9	8 5	8
20	9	18 7	11
20	12	21 2	15
200	11	197	10
2000	9	2232	12

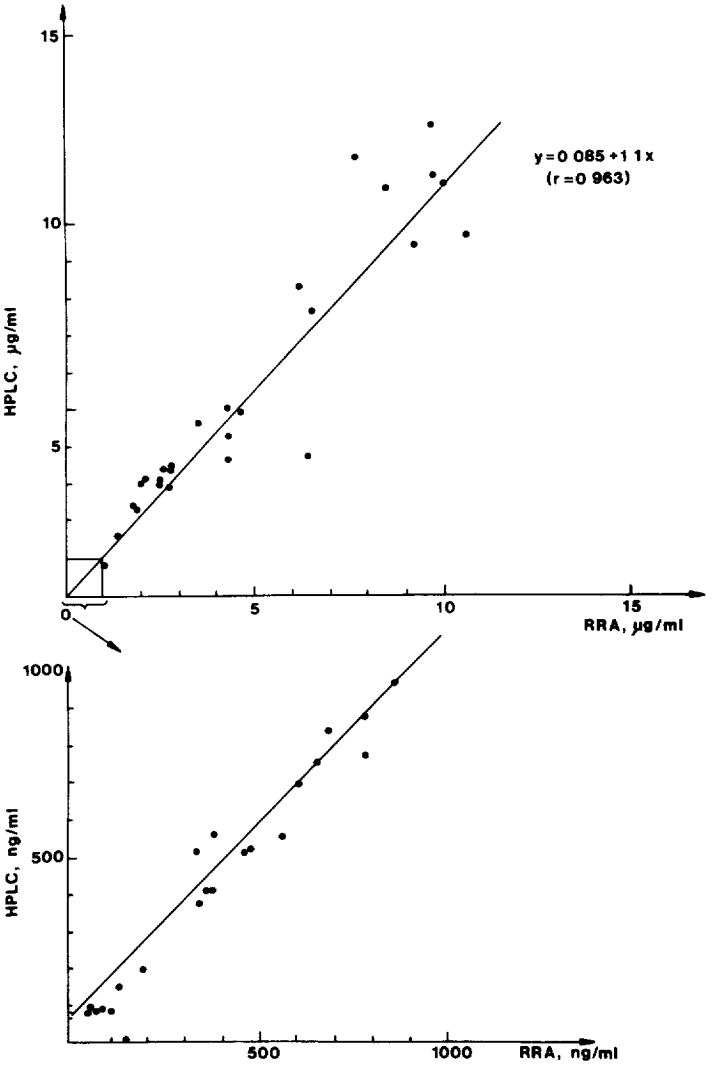


Fig 3 Linear regression analysis of results generated using a benzodiazepine radioreceptor assay (abscissa) and HPLC with UV detection (ordinate) The samples were collected during a 3-month toxicological study in monkeys

25–75% of the maximum binding (i e , binding for standard zero). For I this range is 2 5–20 ng/ml when the sample size is 0 5 ml. With this sample size, the highest sensitivity is 1 ng/ml plasma, corresponding to the detection of an absolute amount of 0.25 ng of I per tube

Recovery and precision The recovery was determined using ^{14}C-labelled I (specific activity 1 52 GBq/mmol) After evaporation, the recovery was 95%

at 2 5 and 20 ng/ml, whereas after redissolving in phosphate buffer the recovery was slightly lower (87–90%) at both concentrations (Table III)

The intra-assay precision was generally below 10%, but at lower concentrations it increased to 18% if the ratio between the activity in the standard zero and in the sample was close to the limit for linearity (Table IV)

The inter-assay precision was investigated using spiked human plasma The precision was around 10%, ranging from 8 to 15% (Table V)

Specificity With the RRA, all substances that bind to the receptor and thus displace [^{3}H]flunitrazepam are measured Therefore, the assay is specific for substances which are ligands for the benzodiazepine receptor, but not specific with regard to compound I Possible active metabolites will consequently be included in the determination if present in the plasma extracts

Accuracy The accuracy of the assay was investigated by comparison with the above-mentioned HPLC method Plasma samples from a 3-month toxicological study in monkeys were assayed using both the RRA and the HPLC method

The concentration of I ranged from 50 ng/ml to 10 μg/ml, and linear regression analysis of the HPLC results as a function of the RRA results gave the regression equation $y = 1\ 11x + 0\ 085$ (Fig 3) The intercept (0 085 μg/ml) was not significantly different from zero ($P > 0\ 6$, t-test), whereas the slope (1 11) was significantly different from unity ($0\ 05 > P > 0\ 02$) This means that the results (uncorrected) generated by the radioreceptor assay in general deviate 10% from the results determined with the HPLC assay

DISCUSSION

Results from two analytical methods for determination of I in plasma from humans, rats and monkeys have been presented Both procedures involve several steps, including extraction The capacity of the RRA procedure is greater than that of the HPLC procedure but, because of a very small linear range for the former, re-analysis of samples is required much more often Hence the HPLC method is to be preferred if only small samples are available and the order of magnitude of the concentration is unknown However, owing to analytical interferences in rat plasma (from endogenous substances) the HPLC method is not suitable for samples from rats

The main drawback of RRA methods is the lack of specificity with regard to both potential active metabolites and also other benzodiazepines which may be measured instead of or together with I

Correction of the RRA results with respect to recovery (approximately 90%) gives a good correlation ($r = 0\ 993$) between the two methods, the regression equation being $y = 0\ 99x + 0.070$ As is evident from Fig 3 (upper), there are some variations between the results from the two methods at higher concentrations (> 5 μg/ml) However, there is no sign of systematic errors The rea-

son for these variations has not been investigated, because the concentration range is not of interest for our future work

The results obtained with the two methods are in good agreement, which strongly indicates that no metabolite with significant benzodiazepine receptor activity is formed in the body.

In conclusion, the RRA is an attractive method for the rapid determination of compound I in plasma samples, but if greater specificity is required the HPLC assay is to be preferred

ACKNOWLEDGEMENTS

The authors thank Mrs U. Laursen and Mr O Wassmann for skilful technical assistance and Mrs L Jensen for preparing the manuscript

REFERENCES

1 J Lund, Scand J Clin Lab Invest , 41 (1981) 275
2 T B Vree, A M Baars, L H D Booij and J J Driessen, Arzneim -Forsch , 31 (1981) 2215
3 U Timm and M Zell, Arzneim -Forsch , 33 (1983) 358
4 C V Puglisi, J Pao, F J Ferrara and J A F de Silva, J Chromatogr , 344 (1985) 199
5 R G Lister, D J Greenblatt, D R Abernethy and S E File, Brain Res , 290 (1984) 183
6 C V Puglisi, F J Ferrara and I Bekersky, J Chromatogr , 417 (1987) 347
7 P J Howard, E McClean and J W Dundee, Anaesthesia, 40 (1985) 664
8 D J Greenblatt, A Locniskar, H R Ochs and P M Lauven, Anesthesiology, 55 (1981) 176
9 C D Syracuse, B R Kuhnert, C J Kaine, A C Santos and M Finster, J Chromatogr , 380 (1986) 145
10 P Heizmann, J -C Jordan and B Ludvig, J Chromatogr , 415 (1987) 170
11 M Zell, E Weidekamm and U Timm, in P Sandra (Editor), Proceedings of the 6th International Symposium on Capillary Chromatography, Huthig, Heidelberg, 1985, p 584
12 F Rubio, S Chen, T Crews, F deGrazia, W A Gorland and M Barbalas, J Chromatogr , 421 (1987) 281
13 J A Glaser, D L Foerst, G D McKee, S A Quave and W L Budde, Environ Sci Technol , 15 (1981) 1426