Journal of Chromatography, 342 (1985) 221—227
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2615

Note

Determination of tiodazosin concentrations in human plasma with a fluorescence high-performance liquid chromatographic method

S. THOMAS FORGUE*, DONALD R VAN HARKEN and ROBERT D. SMYTH

Metabolism and Pharmacokinetics Department, Pharmaceutical Research and Development Division, Bristol-Myers, P.O. Box 4755, Syracuse, NY 13221-4755 (U.S.A.)

(First received December 19th, 1984; revised manuscript received February 19th, 1985)

Tiodazosin is a novel, oral antihypertensive agent that is a chemical and pharmacological analogue of prazosin (Minipress®, Pfizer). Tiodazosin is the levulinate salt of the compound shown in Fig. 1. Both antihypertensives are dimethoxy aminoquinazolines that are highly fluorescent in acidic solutions. This property has been used to develop sensitive and specific high-performance liquid chromatographic (HPLC) assays for prazosin in plasma [1—7]. Recently, an HPLC—fluorescence detection assay method for tiodazosin in plasma has been reported by Mico et al. [8]. Their method entails the addition of prazosin as an internal standard, and injection of samples onto a C_{18} column after deproteinization and partial evaporation under nitrogen. The linear range of the assay was reported to be 6—868 ng/ml. The method described herein entails the use of an internal standard (I.S.) that, unlike prazosin, elutes after tiodazosin, and involves a simple extraction procedure to produce a matrix

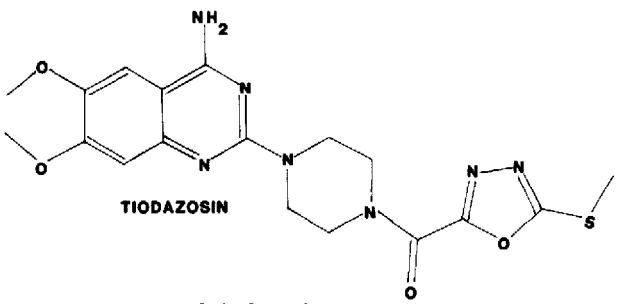

Fig. 1. Structure of tiodazosin.

that is essentially devoid of interference. These features facilitate quantification of 1 ng/ml levels with defined accuracy, as well as detection of fluorescent tiodazosin metabolites.

EXPERIMENTAL

Chemicals

Tiodazosin base, 4-amino-6,7-dimethoxy-2-[4-(5-methylthio-1,3,4-oxadiazole-2-carbonyl)piperazin-1-yl] quinazoline, was used as the reference standard. HPLC-grade solvents were obtained from either Baker or Burdick & Jackson Labs., and other chemicals were of reagent grade. Water was purified with a Milli-Q (Millipore) system. Control human serum was obtained from healthy, drug-free, male volunteers (Interstate Blood Bank). The internal standard used was 4-amino-6,7-dimethoxy-2-[4-(cyclopentyl-carbonyl)-piperazin-1-yl] quinazoline hydrochloride (Bristol Labs., BL-5368A).

Sample processing

Plasma was prepared from fresh heparinized blood by centrifugation (1000 *g*, 5 min), and was stored at −20° C. On the day of assay, samples were thawed and, if necessary, diluted into the standard curve range with control human serum. Each 1.0-ml sample was spiked with 0.15 ml ammonium phosphate (0.50 *M*, pH 8.3) that contained 15 ng I.S. Subsequently, 4 ml methylene chloride were added, and each sample tube was capped, mixed on a rotating rack (Rotorack, Fisher Scientific) for 5—10 min, and centrifuged (2000 *g*, 5 min). The upper, aqueous layer and interface material was aspirated to waste. The organic phase was transferred to a clean tube, and evaporated to 1 ml, under nitrogen, in a water bath (30—35° C). After the addition of 0.40 ml phosphoric acid (36 m*M*), each tube was vortexed, and centrifuged (2000 *g*, 5 min), and a portion of the upper, acid layer containing the drug was transferred to an injection vial insert.

On each assay day, analytical standards containing 1.0—200 ng/ml were prepared, in duplicate, by spiking 1.0 ml serum with 2—80 μl of stock solutions containing 5.0 or 0.50 μg/ml tiodazosin in methanol−25 m*M* hydrochloric acid (1:9). Stock solutions were held at 4° C, and were discarded after one week. Duplicate 1.0-ml serum portions were included in each assay sequence as blank quality control samples (blank QCs). During clinical sample analyses, serum QCs spiked with tiodazosin were prepared, stored, and assayed with study samples to assess the accuracy and precision of each analysis.

Chromatography and data processing

HPLC equipment was obtained from Waters Assoc. unless stated otherwise. Injections of 25 μl were made with a WISP 710B automatic injector. The mobile phase of methanol—4 m*M* sodium phosphate, pH 3.6 (46:54) was degassed with sonication under vacuum, and pumped (Model 6000A; eluate recycled) through a μBondapak C_{18} column (30 × 0.39 cm; 10-μm particles) at a flow-rate of 1.5 ml/min. A Schoeffel FS970 fluorescence detector (Kratos) equipped with a deuterium lamp was operated with these settings: 250 nm excitation, 389 nm cut-off emission filter, 0.02 μA range, 3.00 sensitivity, and 4 sec time constant. The 0—10 mV analog signal from the detector was

recorded both with a Model 1200 chart recorder (Linear Instruments) and, after digitization, with a Model 3356 computer (Hewlett-Packard) to obtain retention times, and peak signals for tiodazosin and the I.S. Peak height ratios (tiodazosin peak height/I.S. peak height) were calculated for each injection. The least-squares linear regression of peak height ratio on concentration for each standard, weighted by the inverse of each standard's nominal concentration, was calculated, and unknown concentrations were estimated by inverse prediction from this function. All tiodazosin concentrations were expressed in terms of the free base per ml plasma.

Assay validation

Recovery of tiodazosin in the extraction procedure was determined by measuring peak heights for extracted samples containing 100 ng/ml of plasma, relative to those for direct injections of standard solutions. Since the final extract volume was 0.4 ml for each 1.0-ml plasma sample, a dilution factor of 0.4 was introduced into the calculation.

Assay accuracy and intra-assay (within-day) precision were assessed by assaying, in a blinded manner, ten replicate samples of two different spiked plasma pools. The deviation of the mean predicted concentration from nominal was taken as an index of accuracy, and intra-assay precision was expressed as percent relative standard deviation (% R.S.D.) for the ten replicates. Additional 1.0-ml portions of each pool were frozen, and on three subsequent days ten replicates of each were assayed to assess inter-assay (between-day) precision. The results for each assay sequence (treatment) were analyzed by ANOVA to obtain the treatment mean square (TrMS), error mean square (EMS), and grand mean (GM). Inter-assay precision was calculated as $100[(\mathrm{TrMS} - \mathrm{EMS})/n]^{0.5}/\mathrm{GM}$, with $n = 10$ replicates.

The degree of interference caused by endogenous constituents was assessed subjectively by inspection of chromatograms for blank plasma samples. Endogenous interference with the tiodazosin peak was also assessed by determining mean predicted concentrations for blank plasma and for spiked (1.0 ng/ml) plasma samples from ten different individuals. These spiked samples were used to establish 1.0 ng/ml as the lower limit of quantitation, by determining the accuracy with which they were assayed. Possible chromatographic interference by putative tiodazosin metabolites was investigated by determining the retention times of the authentic compounds after direct injection of aqueous solutions.

Stability during storage (−20°C) was assessed with 1.0-ml plasma samples containing 100 or 200 ng/ml. Five replicates were thawed and assayed for each analysis. Additional stability samples were incubated at 37°C for 5 min, and then at 25°C for 1, 2 or 4 h prior to freezing to assess stability during study sample collections. To exclude the possibility that tiodazosin is degraded at an appreciable rate in pH 8.3 buffer, a 30-min hiatus at 25°C was introduced into the processing procedure for five test samples. Predicted concentrations were compared to those for five paired samples that were processed without interruption. The concentration of a possible amide hydrolysis product in these incubated samples was estimated by spiking analytical standards with the authentic piperazine derivative such that its concentration was one fifth that of tiodazosin, and by processing peak height data as described above.

Stability in the injection solvent (36 m*M* orthophosphoric acid) was studied by comparing the mean predicted concentration for ten replicate samples that were injected immediately after processing, with the mean for ten replicates that were extracted 30 h before injection. Statistical significance was assessed by a *t*-test for paired samples

RESULTS AND DISCUSSION

The fluorescence response was observed to be a linear ($r > 0.990$) function of concentration from 1 to 200 ng/ml. The recovery of the drug in the processing procedure was approximately 78%. HPLC retention times for tiodazosin and the I.S. were nominally 6 and 10 min, respectively (Fig. 2). No unknown peaks with longer retention times, i.e. late-eluting peaks, have been observed. The lower limit of quantitation was established by inspection of chromatograms for blank and spiked (1.0 ng/ml) plasma samples from ten control subjects. Chromatograms of blank samples were essentially devoid of interference, with a single exception. The anomalous sample, which produced a broad peak equivalent to about 2 ng/ml tiodazosin, was rejected as spurious.

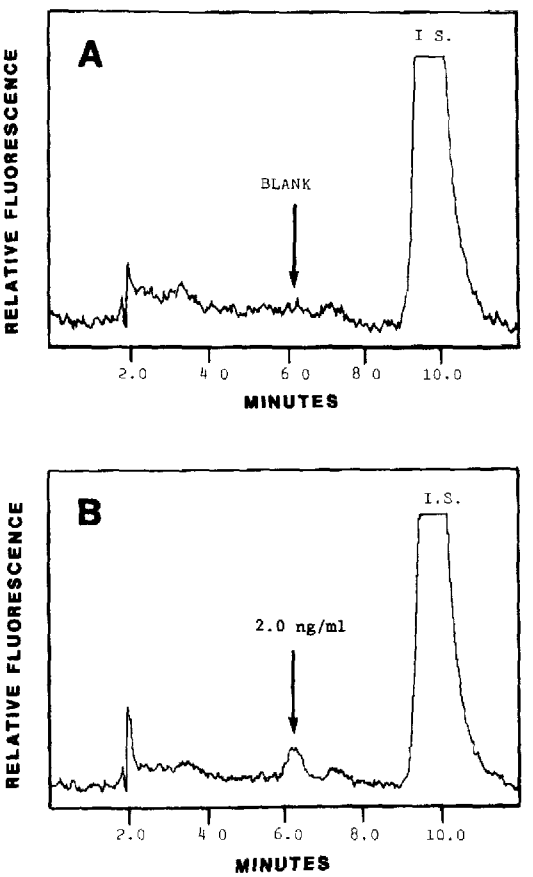

Fig. 2. HPLC profiles of. (A) blank human plasma; and (B) plasma spiked with 2 ng/ml tiodazosin. Hewlett-Packard computer recording.

The mean predicted concentration for the spiked samples was 1.2 ng/ml (R.S.D. 23%), a result indicative of the absence of substantial endogenous interference.

This assay method appears specific for unchanged drug. Compounds tentatively identified as tiodazosin metabolites have retention times different from that of the parent drug (Table I). The predominant tiodazosin metabolism route is via hydrolysis of the amide to produce an unstable, non-fluorescent acid moiety, and 4-amino-6,7-dimethoxy-2-(1-piperazinyl)quinazoline.

Accuracy and precision were assessed by analyzing spiked plasma pools in a blinded manner (Table II). Each pool was subsequently reassayed on three occasions to obtain inter-assay precision values, which were 7.2% and 3.6% at 24 and 160 ng/ml, respectively.

Tiodazosin is stable in serum at −20°C. Mean (% R.S.D.) values for 100 ng/ml samples stored for 35 and 36 weeks were 105 ng/ml (1.2%) and 112 ng/ml (1.9%), respectively. Samples containing 200 ng/ml were stored for 42 weeks,

TABLE I

ASSESSMENT OF ASSAY SPECIFICITY WITH RESPECT TO METABOLITES/ DEGRADATION PRODUCTS AND PRAZOSIN

Compound	Nominal retention time (min)
4-Amino-6,7-dimethoxy-2-(1-piperazinyl)quinazoline	1.9
2,4-Diamino-6,7-dimethoxyquinazoline	2.6
6-O-Demethyltiodazosin	3.8
7-O-Demethyltiodazosin	5.0
Prazosin	5.5
Tiodazosin	6.1
Internal standard (I.S.)	9.7

TABLE II

ASSESSMENT OF ASSAY ACCURACY, AND PRECISION

Spiked serum pools were initially assayed in a blinded manner to assess accuracy and intra-assay precision. The assay procedure was repeated on three subsequent days to assess inter-assay precision. $n = 10$.

Nominal tiodazosin concn. (ng/ml)	Mean observed concn. (ng/ml)	R.S.D. (%)	Deviation from nominal (percentage nominal)
24	23*	4.0	−4.2
	20	4.1	−16.7
	23	3.5	−4.2
	21	5.3	−12.5
160	155*	3 7	−3.1
	149	1.5	−6.9
	158	2.5	−1.2
	146	2.1	−8.8

*Initial assay was blinded.

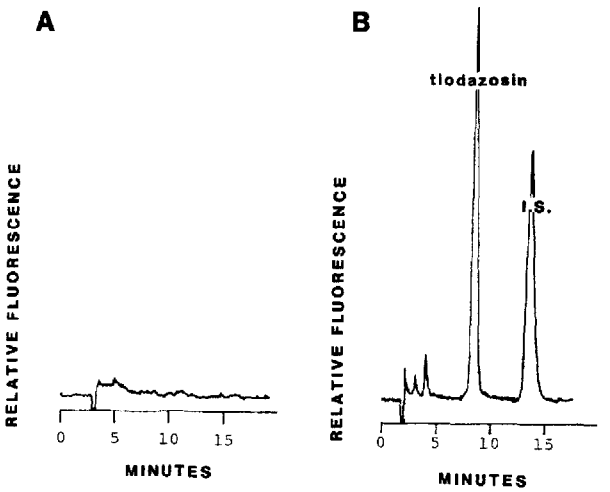

Fig. 3. HPLC profiles from a clinical study assay sequence: (A) control plasma, I.S. omitted; (B) plasma collected 4 h after dosing from the subject described in Fig. 4. Unidentified metabolite peaks are evident in this chart recording.

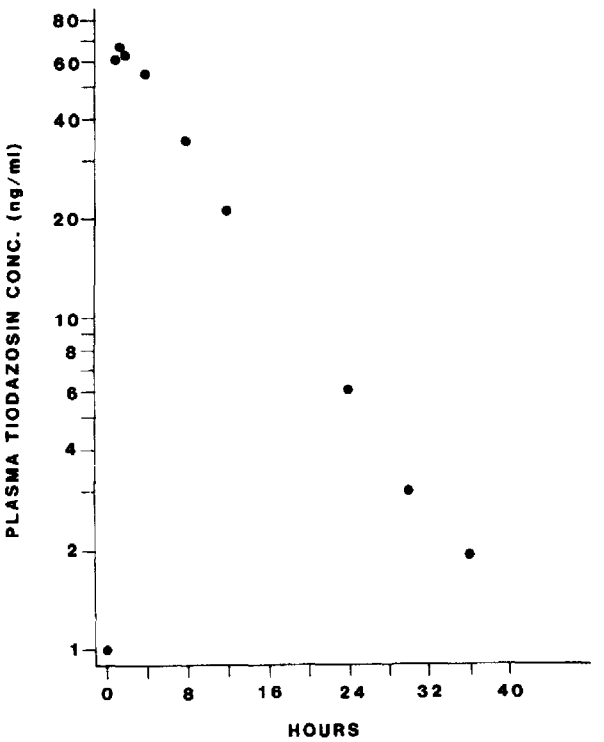

Fig. 4. Plasma tiodazosin concentrations versus time after oral administration of a 5-mg tablet to a male subject who had previously received multiple 2-mg doses. A 48-h wash-out period preceded the 5-mg dose.

and the deviation of the mean observed concentration from nominal was 1.7%. The drug was also stable in the injection solvent; the difference between mean assay values for 160 ng/ml samples held at 23°C for 0 h and for 30 h prior to injection was not statistically significant. Possible hydrolysis of tiodazosin under conditions that might be expected to occur during collection and processing was also investigated. Observed concentrations in samples incubated for 5 min at 37°C, and then 1, 2 or 4 h at room temperature were within 1% of concentrations in paired, non-incubated samples. Incubation at 23°C for 0.5 h after the addition of pH 8.3 buffer had no effect; assay values were 99 and 101 ng/ml for incubated samples and controls, respectively. All of the incubation samples were essentially devoid (< 1 ng/ml) of the piperazine hydrolysis product.

The absence of matrix interference, and the use of an I.S. that elutes after the drug peak facilitates detection of the presence of metabolites, which are relatively non-polar, in individual plasma samples (Fig. 3). Simultaneous quantitation of metabolites in clinical study samples can be accomplished by incorporating the synthetic compound in assay standards in a fixed proportion to the tiodazosin level. The use of prazosin as an I.S. [8] is non-ideal, since prazosin elutes from C_{18} phases immediately before tiodazosin, and is not well resolved from it. This HPLC assay method with fluorescence detection has proven sensitive, accurate and reliable for the determination of plasma tiodazosin concentrations (Fig. 4) in clinical pharmacokinetic studies.

ACKNOWLEDGEMENT

We thank Jeannine L. Briedis for her excellent technical assistance.

REFERENCES

1 T.M. Twomey and D.C. Hobbs, J. Pharm. Sci., 67 (1978) 1468.
2 Y.G. Yee, P.C. Rubin and P. Meffin, J. Chromatogr., 172 (1979) 313.
3 P.A. Reece, J. Chromatogr., 221 (1980) 188
4 E.T. Lin, R.A. Baughman, Jr. and L.Z. Benet, J. Chromatogr , 183 (1980) 367.
5 M.K. Dynon, B. Jarrot, O. Drummer and W.J. Louis, Clin. Pharmacokinet., 5 (1980) 583.
6 J. Dokladalova, S.J. Coco, P.R. Lemke, G.T. Quercia and J.J. Korst, J. Chromatogr., 224 (1981) 33
7 V.K. Piotrovskii, V.G. Belolipetskaya, A.R. El'man and V.I. Metelitsa, J. Chromatogr., 278 (1983) 469.
8 B.A Mico, R.A. Baughman, Jr. and L.Z. Benet, J. Chromatogr., 230 (1982) 203.