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Determination of turosteride, a new inhibitor of 5α-reductase, in human plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

A sensitive and specific HPLC method for the determination of turosteride in human plasma was developed and validated. Turosteride was extracted from plasma with diethyl ether. Further purifications of the fraction extracted were performed sequentially by solid-phase extraction using a CN cartridge and by liquid–liquid partition between *n*-hexane and acetonitrile. Finally the acetonitrile solution containing the test compound was evaporated to dryness and the residue dissolved in the mobile phase, then injected onto the HPLC system. The chromatographic separation was performed isocratically by a reversed-phase column filled with ODS using a water–acetonitrile–methanol mixture. The eluate was monitored at 210 nm. No peak interfering with that of turosteride was observed when blank human plasma was assayed. Linearity was obtained in the turosteride concentration range of 5–1000 ng/ml plasma. Six calibration curves in plasma prepared and run on six different days showed correlation coefficients higher than 0.99 and good reproducibility of the slope (C.V.=4.3%). The intra-day precision, evaluated at three concentrations (in the low, mid and high range of the standard curve) and expressed as C.V., ranged from 0.81 to 13.25%. The inter-day precision evaluated at the same concentrations was better than 10.7%. The inter-day accuracy evaluated in the same samples and expressed as the ratio of found/added amount of turosteride ranged from 97.66 to 98.38%. The limit of quantitation was 5 ng/ml plasma. The HPLC method described was successfully employed for the determination of turosteride in plasma samples obtained during a phase I clinical trial with the test compound.

Keywords: Turosteride; 5α-Reductase

1. Introduction

Turosteride [1-(4-methyl-3-oxo-4-aza-5α-androstane-17β-carbonyl)-1,3-diisopropylurea, code name FCE 26073, TUR, Fig. 1] is an inhibitor of testosterone 5α-reductase [1], the enzyme catalyzing the conversion of testosterone into the potent tissue specific androgen 5α-dihydrotestosterone (DHT). The activity of this enzyme has been recognized as possibly responsible for the pathogenesis of benign prostatic hyperplasia (BPH) [2]. This condition, related to aging, is common to most males aged over 60 and may result in bladder outlet obstruction and recurrent urinary infections.

Common therapy for symptomatic BPH is transurethral prostate resection, but this surgical treatment, although characterized by low mortality and moderate morbidity, often leads to postoperative

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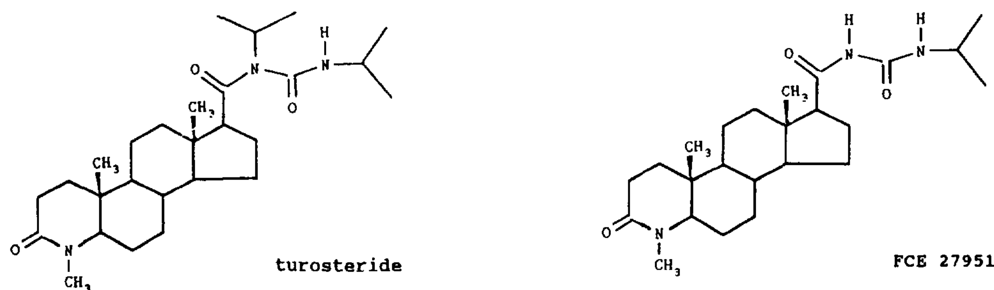

Fig. 1. Structural formulae of TUR and its metabolite (FCE 27951).

complications, mainly in elderly subjects [3,4]. The occurrence of impotence and incontinence after surgical treatment is infrequent but significant since it considerably worsens the patient's quality of life [4].

Non-surgical options and medical treatment therefore appear attractive for BPH management. In particular, specific inhibition of 5α-reductase is considered a valuable pharmacological tool for DHT-mediated disorders (included BPH) since the main testosterone-mediated effects would be preserved [5,6].

Finasteride was the first 5α-reductase inhibitor used for therapy of symptomatic BPH [7]. TUR was synthesized in a research program aimed at obtaining new and selective 5α-reductase inhibitors. In in vitro studies carried out with homogenates of human benign prostatic hypertrophy tissue or of adult rat prostate the compound showed IC_{50} values of 55 and 53 n*M*, respectively [1]. In addition TUR was found to be approximately ten-fold more potent in inhibiting type 2 (IC_{50} 18.3 n*M*) than type 1 human 5α-reductases [8]. TUR showed no relevant binding affinity to the rat prostate androgen receptor or to the estrogen, progesterone, glucocorticoid and mineral-corticoid receptors [9].

In an in vivo study in the immature castrated male rat treated daily with s.c. testosterone propionate alone or concurrently with TUR at oral doses of 1, 3 and 10 mg/kg per day for seven consecutive days, the compound showed a significant effect already at 3 mg/kg per day, the hypertrophic response of testosterone being reduced by 40–50% [1].

Because of its interesting pharmacological activity, TUR is now under development and an analytical method able to determine TUR in plasma was therefore required for phase I studies involving pharmacokinetic evaluation. A RIA method was previously described [10], which was sensitive and specific as indicated by the cross-reaction of the antiserum with some structurally related compounds. However, since a quali-/quantitative picture of the biotransformation compounds circulating in plasma of different animal species following drug administration was not yet available, the setting up of a chromatographic method for the determination of the unchanged drug in this bio-fluid appeared desirable. Chromatographic methods that provide adequate sensitivity are generally preferable to RIA methods and more easily applicable since they do not involve the use of radioactive labels. Thermal instability of TUR discouraged the use of GLC, so our efforts were directed towards the setting up of an analytical method based on HPLC. TUR has low absorption and UV detection could be performed only at low wavelength (210 nm). Since TUR is neither fluorescent nor electroactive, fluorimetric and electrochemical detection could not be used for sensitive determination. In addition the compound did not possess any structural feature that could readily be chemically modified to form a derivative with enhanced detectability. UV detection therefore appeared to be the only detection technique for the quantitative determination of this compound after HPLC separation. Unfortunately, the first assays revealed that several blank plasma constituents could interfere with TUR determination, therefore a selective multi-step extraction had to be applied before the HPLC separation for sample clean-up.

When suitable conditions for the extraction and analysis were established, they were applied to the analysis of plasma samples obtained after oral ad-

ministration of the test compound to male rats. In these samples TUR appeared to be extensively metabolised [11] and some metabolites were found to interfere with the determination of the unchanged drug. New conditions were therefore sought for the separation of the unchanged drug from these unexpected metabolites. Finally suitable conditions for the specific determination of the parent compound were identified and successfully employed for the analysis of plasma samples from preclinical and clinical studies with TUR. The detailed description of this method and its validation in human plasma are presented together with an example of application in a clinical study.

2. Experimental

2.1. Chemicals

TUR (strength higher than 99% as determined by HPLC) was obtained from the Pharmaceutical Development Department of Pharmacia. All reagents and solvents were analytical or HPLC grade from Carlo Erba Reagents (Milan, Italy). Bakerbond CN cartridges (100 mg) were from Baker (code 7021-01, Phillipsburg, NJ, USA).

2.2. Chromatographic equipment

The HPLC system used in this study consisted of a binary gradient pump (Model P2000, Thermo Separation Products (TSP), San Josè, CA, USA), an autosampler (Model AS 3000, TSP) with 100-μl loop, a variable-wavelength UV detector (Model UV 975, Jasco, Tokyo) and a recorder-integrator (Model SP 4270, TSP) connected to a Labnet network (TSP). The detector was set at 210 nm and wired to send a 1-V signal to the integrator.

2.3. HPLC conditions

The chromatographic separation was performed with a 250×4 mm I.D. Erbasil S 3 C_{18} reversed-phase column (particle size 3 μm) (Carlo Erba Reagents) equipped with an Erbasil precolumn (30×4 mm I.D.) filled with ODS (particle size 3 μm).

The separation was performed under isocratic conditions, then a gradient step was employed to elute some lipophilic compounds retained by the column. The mobile phase composition was as follows: system A, water–acetonitrile–methanol (38:37:25, v/v); system B, water–acetonitrile (20:80, v/v). The two solvent mixtures were prepared daily and degassed by He sparging. The flow-rate was 0.6 ml/min. At the start of the analysis the mobile phase A was pumped for 35 min, then, within 1 min, the system was changed to 100% mobile phase B which was pumped for 9 min. A further change to 100% mobile phase A was performed within 2 min, followed by a 13-min period of column stabilization under the initial elution conditions. Run time was 60 min.

2.4. Preparation of standards

A standard stock solution of TUR was prepared by dissolving about 7 mg (exactly weighed) of the test compound in 200 ml methanol. From this stock solution five working solutions were prepared weekly in H_2O–CH_3OH (80:20, v/v) at final concentrations of about 5, 10, 100, 400 and 1000 ng/100 μl. Aliquots of these working standards (100 μl) were spiked into blank human plasma (1 ml) and assayed to evaluate the linearity of the method. For the determination of the precision and accuracy aliquots of the working solutions were added to 1 ml of blank human plasma to obtain TUR concentrations of about 15, 150 and 800 ng/ml.

Stock and working solutions were stored at nominal 4°C until use. In these conditions stock solutions of TUR are stable for at least one month.

2.5. Chromatographic performance

The suitability of the chromatographic system was checked before each series of analyses by evaluating the column efficiency, the peak symmetry and the reproducibility of the response; this evaluation was carried out according to U.S.P. (XXIII, 1995, 1776–1777) using the System Suitability Test software supplied by TSP. The column efficiency was expressed as the number of theoretical plates (N): this value had to be higher than 5000. The tailing factor (used to evaluate the peak symmetry) had to be less than 1.5. The reproducibility of the response evalu-

ated by repeated injection ($n=5$) of a standard solution containing ca. 100 ng of TUR had to give a C.V. lower than 5%.

2.6. Analytical procedure

Into a glass stoppered test tube place 1 ml of the plasma sample, add 1 ml of 1 *N* NaOH and extract with 5 ml of diethyl ether (vortex-mix for 1 min). Centrifuge at 1200 *g* for 5 min.

Separate and evaporate the organic phase at 40°C under a stream of N_2. Dissolve the residue with 0.1 ml ethyl acetate and 1 ml *n*-pentane (vortex-mix 1 min) and load the solution into a 100 mg Bakerbond-CN cartridge (preconditioned in sequence with 1 ml CH_3OH, 1 ml ethyl acetate and 1 ml *n*-pentane). Wash the cartridge with a *n*-hexane–cyclohexane mixture (1:1, v/v, 2×1 ml), discard the washings and elute the test compound with 2×1 ml acetone. Evaporate the acetone solution to dryness under N_2 at 40°C. Dissolve the residue in 1 ml acetonitrile and wash the solution with 3 ml *n*-hexane (vortex-mix 1 min).

Centrifuge (1200 *g* for 1 min) and discard the upper phase. Evaporate the acetonitrile solution to dryness under N_2 at 40°C. Dissolve the residue with 135 μl of the HPLC mobile phase and submit 100 μl of the final solution to the chromatographic analysis under the experimental conditions described in Section 2.3.

2.7. Calculations

The integrator determined the peak areas of the analytes. Calibration curves were obtained by plotting the measured peak areas (counts) (y) vs. the analyte concentration in plasma (ng/ml) (x). Weighted linear regression (weighting factor $1/y$) was used to calculate TUR concentration in quality control and unknown samples.

2.8. Evaluation of the extraction recovery

Blank human plasma (1 ml) was spiked with different amounts of the test compound (about 27, 150 and 640 ng) and assayed as described. Peak areas obtained were compared to the peak areas obtained by direct injection of unextracted standards.

3. Results

Under the chromatographic conditions adopted for analysis TUR gives a sharp peak with t_R of about 21 min (Fig. 2A). The poorly selective conditions employed for analyte detection (at 210 nm several plasma constituents have a strong UV absorption) called for a highly selective extraction procedure to isolate TUR from plasma.

The effectiveness of both solid-phase extraction (SPE) and liquid–liquid (L/L) extraction of TUR from plasma was carefully evaluated. Preliminary data on isolate retention in SPE using different modified sorbents showed poor retention of TUR from the plasma matrix. These data and preliminary findings of a plasma protein-binding assay (performed with the labelled compound), showing that TUR was extensively bound to human plasma proteins (M.G. Castelli, unpublished data), suggested that a L/L extraction step should to be introduced, before the SPE. This step is useful both to displace TUR from plasma proteins, and for a preliminary, though incomplete, sample clean-up. Extraction with diethyl ether of plasma performed under alkaline conditions reduced the extent of plasma interferences co-extracted with TUR and for this reason it was preferred to that performed under neutral or acidic conditions; in all cases the recovery of TUR from plasma was almost quantitative.

SPE using a CN-sorbent proved the most selective among those assayed, and, under the conditions adopted, yielded an almost quantitative recovery of TUR. Some minor interferences from plasma requiring further sample clean-up before the chromatographic analysis were still present in the isolate after this step, however.

A further partition between acetonitrile and *n*-hexane of the residue obtained after the SPE finally allowed us to obtain a clean window in the chromatogram where TUR was eluted (Fig. 2B).

As a whole, the complex extraction procedure developed for isolation of TUR from plasma, in spite of the several steps involved, proved satisfactory in terms of reproducibility of the operation, percentage of TUR extracted and purity of the isolate for the subsequent chromatographic analysis (Fig. 2C).

The mean extraction recovery (±S.D.) from human plasma evaluated on three different days at

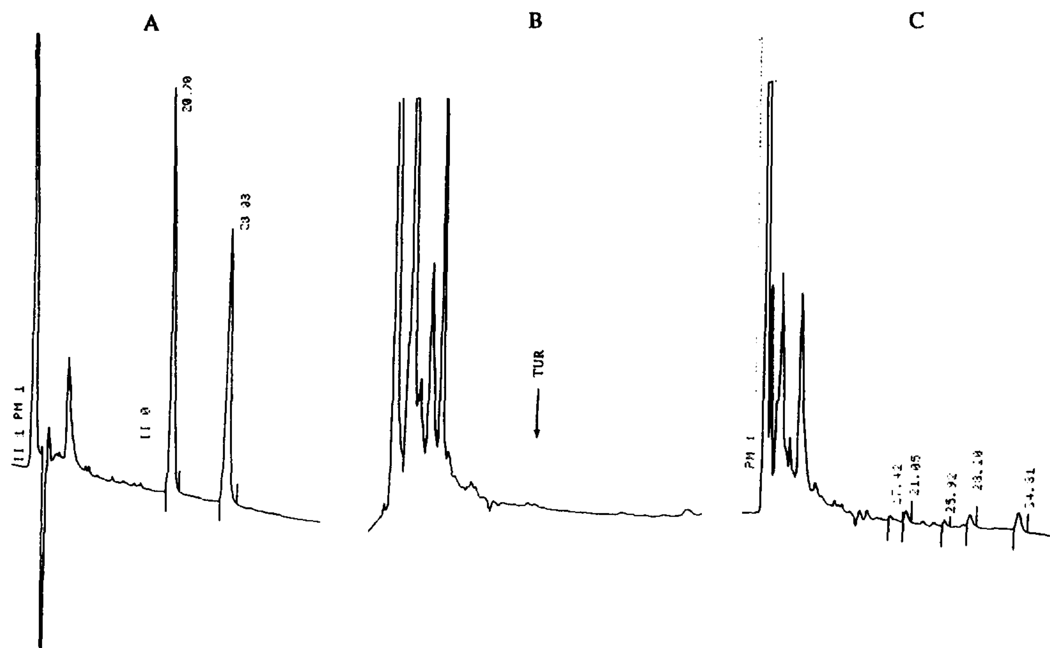

Fig. 2. Chromatograms obtained from a standard solution of TUR (t_R 20.90 min) and its metabolite FCE 27951 (t_R 28.03 min) (A), from 1 ml of blank human plasma (B), and from blank human plasma (1 ml) spiked with 5 ng of TUR and FCE 27951 (C).

27, 150 and 640 ng/ml was 65.3±5.33%, 67.3±4.22% and 67.0±3.08%, respectively (Table 1).

When rat plasma from treated animals was assayed, a drug related peak with t_R higher than that of the unchanged drug (28 vs. 21 min) was observed in the chromatogram together with other peaks with lower retention times (data not shown). All the peaks, however, were well separated from that of the unchanged drug, showing the selectivity of the chromatographic conditions employed in the assay.

A complication in the chromatographic analysis resulted from the presence of some late eluting peaks that were observed from the analysis of blank human and monkey plasma (chromatograms not shown). These peaks, attributable to endogenous components of plasma, required long analysis times (about 90 min) to avoid peak overlapping during the sequential analysis of several bio-samples. This reduced the number of samples per day that could be assayed. To improve the productivity of the assay without affecting its specificity, a modification of the mobile phase composition (see Section 2.3) was introduced after the elution of the last compound of interest (the metabolite having t_R of about 28 min). The rapid change of the mobile phase composition could be easily performed since no buffer was present in the two solvent mixtures (the presence of a buffer was of no value either in the separation of TUR and its metabolites, or in the elution of the other plasma constituents). The effects of the change in the mobile

Table 1
Evaluation of the extraction recovery of TUR from plasma

Turosteride conc. (ng/ml)	Day	n	Recovery (%)	S.D. (%)	C.V. (%)
27.22	1	3	60.14	1.62	
27.22	2	3	66.70	5.76	
27.22	3	3	69.08	3.70	
Mean±S.D.		9	65.31	5.33	8.16
151.2	1	3	66.12	7.31	
151.2	2	3	66.63	2.81	
151.2	3	3	69.10	1.55	
Mean±S.D.		9	67.28	4.22	6.27
642.6	1	3	64.72	1.17	
642.6	2	3	66.20	2.40	
642.6	3	3	70.20	2.60	
Mean±S.D.		9	67.04	3.08	4.59

phase composition were evaluated in terms of reproducibility and performance of the chromatographic separation: the retention time of TUR and its metabolites showed very good intra- and inter-day reproducibility; tailing factor and theoretical plate number did not show appreciable variations. The precision of replicated determinations was unaffected by the change in the mobile phase composition. In addition the life-span of the column proved acceptable since more than a thousand samples could generally be assayed using the same column.

The linearity of the determination was evaluated on six calibration curves which were prepared and run on six different days with TUR in the concentration range of 5–1000 ng/ml plasma. Correlation coefficient (r) for the regression ranged from 0.9927 to 0.9997. The mean calibration curve obtained was described by the equation $y=3468x-3896$ (slope C.V.=4.3%, $n=6$), where y=peak area (counts) and x=TUR amount (ng) added to 1 ml plasma. The back-calculated standard concentrations exhibited a C.V. of less than 9.5% (Table 2).

The precision of replicate determinations of TUR evaluated at three concentrations on six different days is summarized in Table 3. The intra-day precision (expressed as C.V.) ranged from 0.81 to 13.25%. The inter-day precision evaluated at the same concentrations was better than 10.7%.

The accuracy of replicate determinations of TUR evaluated at three concentrations on six different days is summarized in Table 3. Accuracy, expressed as the ratio of found/added amount of TUR ranged from 87.75 to 111.44%. The pooled accuracy (inter-day) ranged from 97.66 to 98.38%.

The limit of quantitation was 5 ng/ml plasma corresponding to about 3.2 ng on column. Spiked samples at this concentration showed a signal-to-noise ratio better than 10:1 and the C.V. of replicate determinations ($n=6$) was less than 8% (Table 2 and Fig. 2C).

The HPLC method described here was employed for the determination of TUR in plasma samples obtained in a phase I clinical study aimed at evaluating the tolerability and preliminary pharmacokinetics of the drug in healthy male volunteers. This study was carried out at the Institut Aster, Hopital Cognacq-Jay (Paris, France) under the responsibility of Dr. J.J. Thebault. The study protocol was approved by the Institutional Ethics Committee and written informed consent was obtained from the subjects who participated in the study. Three subjects received TUR (25 mg, capsules) as a single oral dose. Blood was collected into heparinized tubes at times 0, 1, 2, 4, 8 and 24 h after dosing and plasma separated and frozen until assayed.

The chromatograms obtained from the plasma samples collected at 2 and 8 h after dosing from a subject (Subject 13) are shown in Fig. 3. No peak interfering with that of the unchanged drug was observed, showing the selectivity of the analytical procedure developed. The method was sensitive enough to permit the determination of the unchanged drug in plasma up to the last sampling time (24 h). The mean plasma levels (±S.D.) of TUR obtained at

Table 2
Back-calculated concentrations and standard curve parameters for TUR in human plasma

Validation day	Concentration found (ng/ml)					Slope	y-Intercept	r
	5.03[a]	10.05	100.50	402.0	1005.0			
1	5.09	9.17	98.50	428.3	944.8	3401.8	1108.7	0.9941
2	5.89	9.17	106.91	333.2	1088.7	3679.8	−3914.3	0.9927
3	5.86	10.31	88.34	343.4	1093.8	3596.7	−6108.7	0.9935
4	5.83	9.29	95.44	373.0	1043.2	3314.9	−5887.4	0.9986
5	4.96	11.21	92.93	384.4	1031.3	3489.0	−7664.3	0.9992
6	5.89	9.38	92.37	402.9	1013.1	3328.1	−788.2	0.9997
Mean	5.59	9.76	95.75	377.5	1035.8	3468.4	−3895.8	
S.D.	0.44	0.83	6.43	35.82	54.83	147.8		
C.V.(%)	7.8	8.5	6.7	9.5	5.3	4.3		

[a] Standard concentrations (ng/ml).

Table 3
Accuracy and precision of the method for the determination of TUR in human plasma

Control sample (ng/ml)	Day	n	Accuracy		Precision	
			Mean found (ng/ml)	Recovery (%)	C.V. (%) (intra-day	Pooled C.V. (%) (inter-day
15.08	1	3	14.82	98.25	10.75	
	2	3	13.56	89.95	1.33	
	3	3	13.23	87.75	3.36	
	4	3	14.33	95.00	13.25	
	5	3	16.80	111.44	6.82	
	6	3	15.62	103.56	3.60	10.7
150.80	1	3	155.87	103.36	0.81	
	2	3	147.00	97.48	6.45	
	3	3	147.75	97.98	4.43	
	4	3	149.07	98.85	3.37	
	5	3	147.77	97.99	2.99	
	6	3	142.65	94.59	8.03	4.9
804.00	1	3	776.82	96.62	9.15	
	2	3	724.46	90.11	10.10	
	3	3	792.28	98.54	6.48	
	4	3	849.15	105.62	1.01	
	5	3	784.51	97.58	10.40	
	6	3	792.45	98.56	9.41	8.4

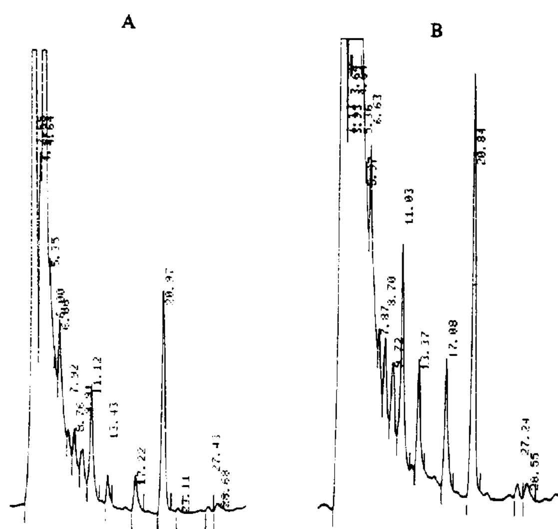

Fig. 3. Chromatograms obtained from the plasma samples collected at 2 h (A) and 8 h (B) after dosing from Subject 13 who had received a single oral dose of TUR (25 mg, 0.2 and 0.5 ml plasma assayed, respectively).

the different sampling times from the three subjects are shown in Fig. 4.

This method was also applied to the determination of TUR in pharmacokinetic and toxicokinetic studies carried out in mice, rats, dogs and monkeys. In all cases no interfering peak at the t_R of TUR was observed when blank plasma was assayed. In addition no metabolite was found to interfere with the determination of the unchanged drug.

In rat and monkey plasma significant concentrations of a metabolite with t_R of about 28 min were found (data not shown): this compound assayed with different columns and mobile phases showed a chromatographic behaviour similar to that of FCE 27951 (Fig. 1), a structurally related compound already synthesized as a possible TUR metabolite [9]. Further confirmation to the identity of this plasma metabolite came from analysis by mass spectrometry which was carried out later on a sample isolated after HPLC assay of rat plasma obtained from treated animals (C. Allievi et al., unpublished data). Since FCE 27951 in in vitro studies proved more active than the parent compound in the rat, but less active in humans [11], its determination in

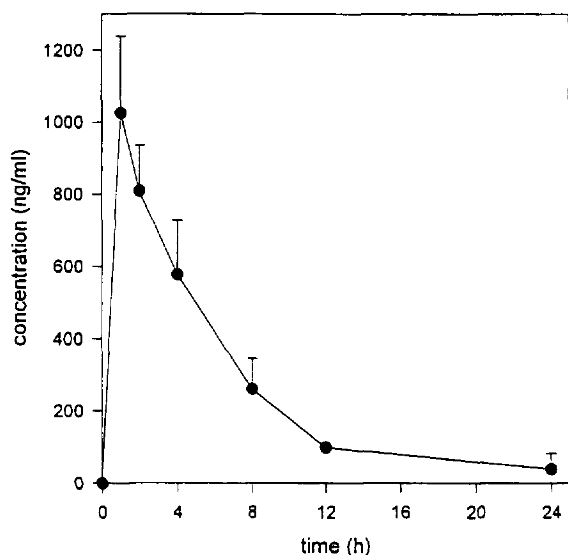

Fig. 4. TUR plasma levels (mean value±S.D.) obtained after a single oral administration (25 mg) to three healthy male volunteers (at 12 h, $n=2$).

plasma appeared desirable in order to correlate pharmacodynamic and pharmacokinetic data.

Unfortunately, calibration and quality control data showed clearly that FCE 27951 could be determined in plasma with less precision and accuracy than the parent compound. This was not fully unexpected since a very selective procedure had been set up for TUR extraction and sample clean-up before the chromatographic analysis. On careful examination of FCE 27951 behaviour in each step of the sample preparation procedure, the poor solubility of the compound in the solvent mixture used to dissolve the residue after the extraction with diethyl ether and before the SPE with the CN cartridge was identified as the crucial point. Some attempts to modify the composition of the solvent mixture used caused a considerable worsening of the sample clean-up or, alternatively, of TUR recovery, thus it was decided that no changes should be made to the analytical procedure described since good characteristics of reproducibility had been obtained for the determination of the parent compound.

In the final conditions chosen for TUR determination, linearity for FCE 27951 could be established in the range 5–1000 ng/ml plasma with values for the slope similar to those obtained for TUR, but with correlation coefficients sometimes slightly lower than 0.99 and with back-calculated concentrations within ±30% of the expected values. Similar variations were found in the assay of quality control samples. Despite these disappointing results, the possibility of estimating the plasma concentrations of the metabolite was useful since it could be established that in humans the relative amount of this metabolite was much smaller than in rats, mice and monkeys.

4. Conclusions

A specific and sensitive analytical method for the determination of TUR in human plasma based on HPLC with UV detection has been reported here. The method proved to be precise and able to quantify the test compound over a range of concentrations of 5–1000 ng/ml plasma. Blank human plasma assayed as described gave interference-free chromatograms at the retention time of the compound of interest showing the selectivity of the method developed (similar results were obtained with blank mouse, rat, dog and monkey plasma). If compared with the RIA method previously developed for TUR, this HPLC method shows less sensitivity, but, since a complete picture of the biotransformation compounds present in human plasma after TUR administration is not yet known, it appears convenient to employ the new HPLC method for the determination of the unchanged drug in the plasma samples collected in clinicals trials. A further advantage of the HPLC method over RIA is the possibility of estimating the levels of a metabolite (FCE 27951) within the same assay. The sensitivity of this HPLC method is suitable for the accurate determination of TUR in plasma following a single oral dose of 25 mg as demonstrated by the preliminary data obtained in a phase I clinical study.

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