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Determination of panomifene in human plasma by high-performance liquid chromatography

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Abstract

An ion-pair HPLC method was developed for the determination of the antiestrogenic drug panomifene, (*E*)-1,2-diphenyl-1-{4-[2-(2-hydroxyethylamino)ethoxy]phenyl}-3,3,3-trifluoropropene, in human plasma. Tamoxifen, 20 ng in 1 ml of plasma, was used as an internal standard. The compounds were isolated from plasma by liquid–solid extraction. Fluorescence detection was achieved by on-line photochemical conversion of the compounds into highly fluorescent phenanthrene derivatives. The sensitivity of the method was 1 ng/ml. The within-day and between-day precision, linearity, extraction recovery and stability of panomifene in plasma and in deproteinized plasma were determined for validation of the method. The method is suitable for measuring plasma levels of panomifene and tamoxifen and for pharmacokinetic studies.

1. Introduction

Panomifene (PAN) (*E*)-1,2-diphenyl-1-{4-[2-(2-hydroxyethylamino)ethoxy]phenyl}-3,3,3-trifluoropropene, is a compound structurally similar to tamoxifen (TMX) (Fig. 1). In preclinical *in vitro* and *in vivo* studies it was found to be a potent antiestrogen with a strong inhibitory effect on estrogen-dependent tumours. The binding affinity to the estrogen receptors of cytosol appeared to be more than double that of TMX. Essential pharmacological activity is associated with proper agonistic and consecutive central (antigonadotropic) actions [1,2]. The favourable properties led to the selection of PAN for a clinical phase I trial.

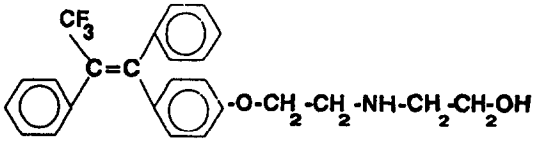

Fig. 1. Structure of panomifene, (*E*)-1,2-diphenyl-1-{4-[2-(2-hydroxyethylamino)ethoxy]phenyl}-3,3,3-trifluoropropene.

Similarly to published HPLC methods for TMX [3–7], an ion-pair chromatographic method was developed for the determination of PAN in human plasma. TMX was used as an internal standard (I.S.). Fluorescence detection was achieved by "on-line" photochemical conversion of the compounds under UV irradiation into highly fluorescent phenanthrene products using a postcolumn photoreactor included in the HPLC system.

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2. Experimental

2.1. Reagents and chemicals

Panomifene (PAN, EGIS-5650) and tamoxifen citrate (TMX) were kindly provided by EGIS Pharmaceuticals (Budapest, Hungary).

Analytical-reagent grade chemicals were used as received: potassium dihydrogenphosphate and 85% phosphoric acid were purchased from Reanal (Budapest, Hungary), triethylamine from Fluka (Buchs, Switzerland), and 96% sulphuric acid from Rudi Pont (Milan, Italy). 1-Heptanesulphonic acid sodium salt (chromatography grade) was obtained from Supelco (Gland, Switzerland) and acetonitrile (LiChrosolv grade) from Merck (Darmstadt, Germany). AMPREP Phenyl (PH) microcolumns (1 ml) were supplied by Amersham (Aylesbury, UK).

Doubly distilled water was used for the preparation of the solutions. Chromatographic eluents were filtered through a Paraplan membrane filter (0.2 μm) before use.

Polypropylene tubes and vials were used for preparing, storing and measuring the standard solutions of PAN and TMX and the biological samples.

2.2. Instruments and equipment

PT 1200 and R 160 P balances (Sartorius, Göttingen, Germany), an OP 208/1 precision digital pH meter (Radelkis, Budapest, Hungary), a Vibrofix (vortex mixer, Janke et Kunkel, IKA, Staufen, Germany), a K23 refrigerated, time-programmable centrifuge (Janetzki, Engelsdorf, Germany), an MPW-310 time-programmable micro centrifuge (Mechanika Precyzyjna Warszawa, Warsaw, Poland) and a freezer (−24°C) (Lehel Jászberény, Hungary) were used.

Solid-phase extraction was carried out using a laboratory-made processing system. The extracts were evaporated under nitrogen with a laboratory-made sample evaporator equipped with a Block-therm thermostat (Kutesz, Budapest, Hungary).

The HP 1084 B HPLC system (Hewlett-Packard, Palo Alto, CA, USA) consisted of a variable-volume injector (79841 A), an automatic sampling system (79842 A) and a terminal (7985 B). A Shimadzu (Kyoto, Japan) RF-530 fluorescence HPLC monitor was used. A Model C 6808 Beam-Boost photoreactor (ICT, Frankfurt, Germany) was incorporated on-line between the chromatograph and the detector.

2.3. Chromatographic conditions

The chromatographic separation was achieved on a 250 mm × 4.6 mm I.D. Si-100-S 10 Phenyl (10 μm) column equipped with a 20 mm × 4.6 mm I.D. SI-100-S Phenyl precolumn (BST, Budapest, Hungary). The mobile phase consisted of 0.005 *M* heptanesulphonic acid in 0.05 *M* KH_2PO_4–H_3PO_4 buffer (pH 3.0) containing 300 μl of triethylamine (TEA) per litre as A and acetonitrile as B eluent, with the composition A–B (25:75, v/v). The flow-rate was 1.2 ml/min. The temperatures of eluents A and B were 80 and 60°C, respectively. The sample injection volume was 10–30 μl.

The postcolumn photochemical derivatization of PAN and TMX was accomplished on-line in a 10 m × 0.3 mm I.D. reaction coil (PTFE tube) knitted on the mercury lamp (UV wavelength 254 nm) of the Beam Boost photoreactor.

The excitation and emission wavelengths of the fluorescence detector were 257 and 378 nm, respectively.

2.4. Sample preparation

Heparinized plasma samples were used. Plasma was separated from whole blood by centrifugation and was immediately stored frozen at −24°C until processing. Before use the plasma samples were thawed at room temperature. Stock standard solutions of PAN and TMX (10.0 mg in 10.00 ml of methanol) were prepared. Working standard solutions containing 1–100 ng of PAN in 20-μl aliquots were prepared by diluting the stock standard solution with methanol–water (1:1, v/v).

Plasma samples (980 μl) were spiked with 20-μl aliquots of PAN working standard solutions, then 20 ng of TMX [in 10 μl of methanol–

water (1:1, v/v)] was added to each plasma sample as an I.S.

The spiked plasma samples prepared in conical polypropylene tubes were processed with 1000 μl of acetonitrile for deproteinization. The centrifuge tubes were capped, mixed on a vortex mixer for 1 min and centrifuged for 60 min at 5000 rpm (2500 *g*) with a free-swinging rotor at −10°C.

A 1600-μl volume of the supernatant was transferred to a calibrated 2 ml polypropylene vial and diluted to volume with 0.05 *M* phosphate buffer (KH_2PO_4–H_3PO_4) (pH 3.0) containing 2% heptanesulphonic acid. The mixture was extracted by liquid–solid extraction using a phenyl microcolumn (1-ml capacity). The columns were conditioned with 5 × 1 ml of acetonitrile and 1 ml of doubly distilled water, followed by 1 ml of 0.05 *M* phosphate buffer (pH 3.0) containing 250 μl of TEA per 100.00 ml.

The deproteinized sample, containing an ion-pair reagent, was transferred on to the previously activated column. After the column had been washed consecutively with 2 × 100 μl of 0.05 *M* phosphate buffer (pH 3.0) containing 0.005 *M* heptanesulphonic acid–acetonitrile (20:80, v/v), 50 μl of 0.025 *M* sulphuric acid was added to adjust the pH of the column. PAN and TMX were eluted with 5 × 100 μl of 0.05 *M* phosphate buffer (pH 3.0) containing 0.005 *M* heptanesulphonic acid–acetonitrile (20:80, v/v).

The eluate was collected in a polypropylene Eppendorf vial and evaporated under nitrogen at ambient temperature. The residue was dissolved in 100 μl of 0.05 *M* phosphate buffer (pH 3.0) containing 0.005 *M* heptanesulphonic acid–acetonitrile (30:70, v/v) and an aliquot was injected into the chromatographic system.

2.5. Validation

Quantitative analysis was performed by the internal standard method, measuring the area of chromatographic peaks. Factors needed for the calculations were obtained by analysis of blank plasma and standard samples supplemented with known amounts of PAN and the I.S.

Standard samples were prepared by adding a 20-μl aliquot of a working standard solution of PAN and 10 μl of the I.S. (20 ng) in methanol–water (1:1, v/v) to 0.05 *M* phosphate buffer (pH 3.0) containing 0.005 *M* heptanesulphonic acid–acetonitrile (30:70, v/v) mixture to give a final volume of 100 μl.

Calibration graphs were fitted by the computer program Medusa Version 1.5/1.587 (CheMicro, Budapest, Hungary). For statistical evaluations a computer program published by Tallarida and Murray [8] was used.

Precision of the chromatographic system

Repetitive injections ($n = 6$) were made of PAN standard solutions of three different concentrations (100, 20 and 5 ng per 100 μl). Each sample contained 20 ng of TMX as I.S. The injection volume was 10 μl.

Linearity, within-day precision

The linearity of the method was studied in a concentration range corresponding to the expected plasma levels with six independent parallel measurements at each concentration. For standard samples 5, 10, 20, 50 and 100 ng per 100 μl and for spiked plasma samples 1, 2, 5, 10, 20, 50 and 100 ng/ml (final volume 100 μl) of PAN were determined. Each sample contained 20 ng of the I.S.

Calibration graphs were constructed by plotting the concentrations of PAN against the peak-area ratios (PAN/I.S.). Statistical analysis of the results was performed to calculate the within-day precision of the method.

Between-day precision

Calibration graphs were prepared by adding PAN to three independent samples of blank plasma at concentrations of 5, 10, 20, 50 and 100 ng/ml on six consecutive days. The study demonstrated both the within-day and the between-day precision.

Recovery

The extraction recoveries of PAN and the I.S. were calculated by comparing the peak areas obtained with spiked plasma samples after extraction with those obtained with standard sam-

ples at the same concentration (independent samples).

Stability of PAN in human plasma

The stability of PAN was examined in human plasma spiked at concentrations of 100, 50 and 10 ng/ml and stored at −24°C for 4 weeks, determinations being made weekly. The stability of PAN in human plasma spiked with the same PAN concentrations deproteinized with acetonitrile was also studied.

3. Results

Without previous photocyclization when the "on-line" photoreactor was switched off, no peaks could be observed with standard solutions containing PAN and the I.S. Typical chromatograms of blank plasma, a standard sample containing 20 ng per 100 μl of PAN and the I.S. and a spiked plasma containing PAN (20 ng/ml) and the I.S. (20 ng/ml) are shown in Fig. 2. The chromatographic system provided a good separation of PAN and the I.S. from the endogenous components. The chromatogram of blank plasma showed no significant matrix interference peaks.

The reproducibility of the chromatographic conditions (retention time, peak area, ratio of the peak areas) was determined by replicate injections ($n = 6$) of 10 μl of standard samples containing 20 ng of the I.S. and 100, 20 and 5 ng of PAN in 100 μl. This examination also tested the reproducibility of the photoconversion of the compounds by using the "on-line" photoreactor.

In this set, the photocyclization depended on the flow-rate of the mobile phase and the length of the irradiated pathway. Owing to the length of the knitted reaction coil (10 m) in a fixed configuration and the applied flow-rate of 1.2 ml/min, the irradiation period was allowed to be *ca.* 50 s according to the instruction manual for the reactor.

Based on the system suitability test, the relative standard deviations (R.S.D.s) for retention times, peak areas and ratio of peak areas were found less than 0.5%, 6% and 7%, respectively. The reproducibilities of the peak shapes, retention times and the photochemical conversions were acceptable under these conditions. On evaluating 20 consecutive chromatograms, the retention time of PAN varied between 5.65 and 5.80 min (5.73 ± 0.05 min) and that of the I.S. varied between 6.95 and 7.12 min (7.03 ± 0.05 min). The retention times increased slightly with ageing of the chromatographic column.

The minimum detectable concentration was 20 pg/ml and the limit of quantification was 1 ng of PAN in 1 ml of plasma. Calibration graphs were constructed to demonstrate the linear relationship between the peak-area ratio and the concentration of the samples. The range covered 1–100 ng/ml for plasma. The upper limit of

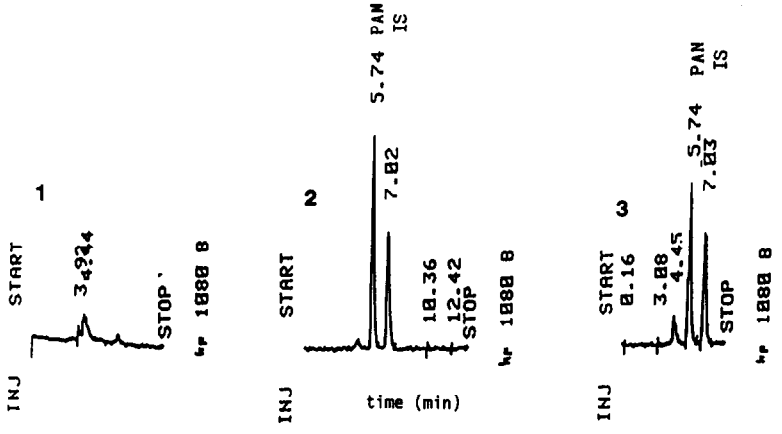

Fig. 2. Typical chromatograms of (1) blank plasma, (2) standard sample containing 20 ng per 100 μl of PAN and I.S. and (3) spiked plasma containing PAN (20 ng/ml) and I.S. (20 ng/ml).

quantification, however, was 300 ng/ml for PAN compared with 20 ng/ml for the I.S. A linear relationship was found in the investigated range with a correlation coefficient (r^2) of 0.999. The intercept was zero. The R.S.D.s (within-day precision) were between 2.89 and 8.90%, which are acceptable values for biological samples.

The reproducibility of the method was demonstrated by the construction of calibration graphs in the concentration range 5–100 ng/ml using three freshly made daily independent parallel plasma samples at each concentration over 6 days. The statistical evaluations of the within-day and the between-day precisions are summarized in Table 1.

Table 1
Within-day and between-day precisions of the assay for panomifene in spiked plasma

Day	*n*	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)
Within-day precision			
1	3	4.847 ± 0.217	4.47
		9.986 ± 0.625	6.25
		20.546 ± 3.046	14.83
		45.055 ± 2.120	4.70
		100.292 ± 5.913	5.89
2	3	5.598 ± 0.527	9.41
		10.842 ± 1.010	9.31
		23.031 ± 3.980	17.28
		49.321 ± 1.270	2.57
		101.279 ± 1.680	1.66
3	3	6.345 ± 0.856	13.49
		12.051 ± 0.542	4.50
		21.696 ± 2.380	10.97
		47.279 ± 2.162	4.57
		102.039 ± 3.907	3.83
4	3	5.204 ± 0.822	15.78
		10.299 ± 0.336	3.26
		20.782 ± 0.628	3.02
		49.756 ± 1.434	2.88
		100.399 ± 2.193	2.18
5	3	5.486 ± 0.211	3.85
		9.565 ± 0.121	1.26
		19.412 ± 0.761	3.92
		51.273 ± 3.524	6.87
		99.592 ± 2.839	2.85
6	3	5.891 ± 0.629	10.67
		10.026 ± 1.014	9.87
		19.883 ± 1.763	8.86
		52.886 ± 2.107	3.89
		99.610 ± 2.390	2.40
Between-day precision			
–	18	5.584 ± 0.682	12.21
		10.462 ± 1.014	9.69
		20.892 ± 2.370	11.35
		49.262 ± 3.145	6.39
		100.535 ± 3.041	3.02

Table 2
Extraction recoveries of panomifene and the internal standard

Compound	Plasma concentration (ng/ml)	Extraction recovery [mean ± S.D. ($n = 6$)] (%)
PAN	100	74.36 ± 13.60
	50	77.50 ± 12.37
	20	56.89 ± 8.29
	10	69.41 ± 11.55
	5	67.21 ± 16.68
I.S.	20	76.42 ± 13.70

Data concerning the extraction recoveries of the compounds are presented in Table 2. The extraction of PAN and the I.S. was not complete but was sufficiently high to determine the concentrations of PAN at low ng/ml levels in plasma.

During 4 weeks of storage at −24°C, the concentration of PAN in the plasma decreased by about 50%; however, no degradation product could be seen in the chromatogram. No significant decrease in concentration was observed in plasma deproteinized with acetonitrile (Fig. 3).

The method has proved to be sufficiently sensitive and selective to be used in clinical pharmacokinetic studies. Fig. 4 shows a typical plasma concentration–time curve after oral administration of 24 mg of panomifene to a healthy female volunteer.

4. Discussion

The HPLC method described here for the determination of PAN and TMX (I.S.) is a sensitive and rapid procedure suitable for clinical and research use.

Methods proposed previously to determine TMX and TMX analogues in biological fluids use fluorimetric detection based on measurement of

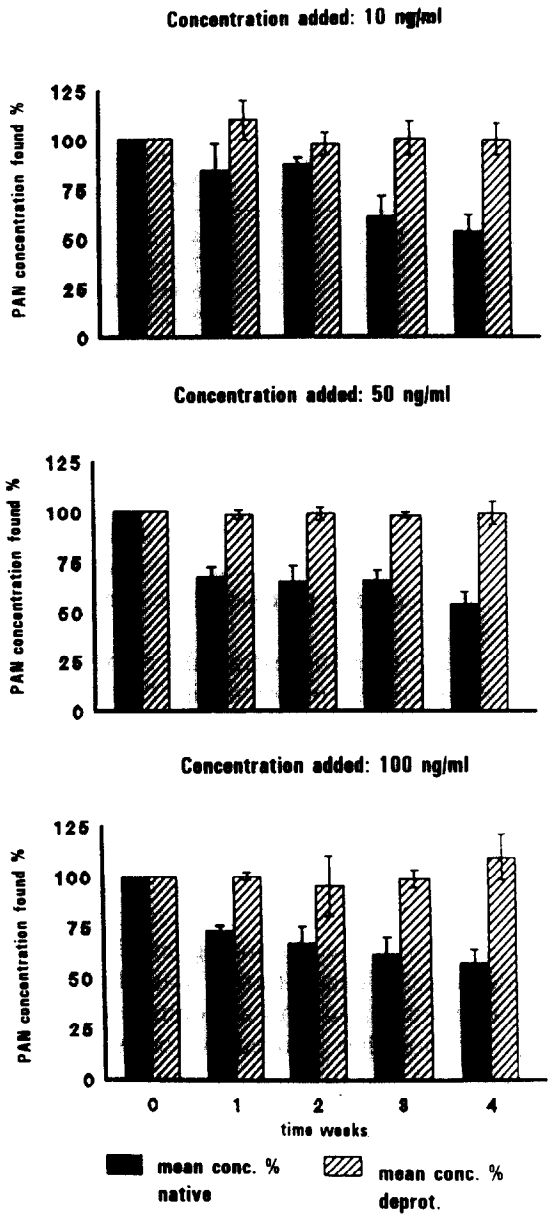

Fig. 3. Stability of PAN in native plasma and in plasma deproteinized with acetonitrile. Time of storage, 4 weeks; temperature of storage, −24°C. A 20 ng/ml concentration of the I.S. was added to each sample before examination.

the fluorescence developed by UV photochemical conversion of the triphenylethylene nucleus to phenanthrenes [3–7]. With "off-line" photolysis and injection of the photocyclization products into the chromatographic system, we were

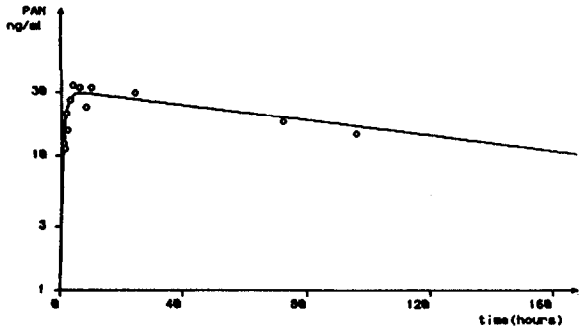

Fig. 4. Plasma concentration–time curve for PAN after oral administration of 24 mg of panomifene to a healthy female volunteer. Plasma half-life of absorption: $t_{1/2}(k_a) = 0.91$, plasma half-life of the elimination of the drug: $t_{1/2}(k_e) = 103.95$, lagtime (time elapsed between drug administration and appearance of the drug in the plasma): $t_{lag} = 0.50$ h. Peak of the plasma concentration: $C_{max} = 29.52$ ng/ml; time to peak = 6.79 h. Area under the plasma concentration–time curve from 0 to ∞ calculated by trapezoidal rule: $AUC_{0-\infty}$ (trap.rule) = 4714.79 (ng/ml)·h; mean residence time: MRT = 153.4 h.

not able to achieve a good resolution for the corresponding PAN phenanthrene derivative. Postcolumn fluorescence activation developed by Brown *et al.* [4] overcame this problem.

Using postcolumn on-line photocyclization, the exposure to UV radiation was dependent only on the stability of the flow-rate and the UV lamp intensity. The geometry of the system was fixed. To achieve the maximum photochemical conversion of PAN, an illumination period of 50 s at a flow-rate of 1.2 ml/min was required, which corresponded to 10 m of illuminated reaction coil. Increasing the illumination period decreased the formation of the fluorescent products.

C_8 and C_{18} reversed-phase columns and mobile phases with a high percentage of organic modifier [3] showed strong peak tailing for the compounds studied. This could be minimized, but not eliminated, by adding an organic amine [4] or an ion-pairing substance [3] to the mobile phase. Similarly to Camaggi *et al.* [5], we were not able to elute TMX from a LiChrosorb 5 RP-18 (Chrompack) column (150 × 4.6 mm I.D.) even by using acetonitrile–0.005 *M* pentanesulphonate (pH 3.0) (80:20) as the mobile phase.

Complete chromatographic separation and resolution of PAN and TMX with a cyano-bonded stationary phase [5] and acidified aqueous acetonitrile containing an ion-pairing reagent as eluent could not be reproduced.

Adequate separation of the analytes and plasma constituents was achieved with a phenyl column eluted isocratically with acetonitrile–phosphate buffer containing heptanesulphonate. As expected, a decrease in pH led to a decrease in the retention times of the basic compounds. The optimum operating pH range in our system was 3.0. TEA could be seen as a tailing-suppressing agent, which improved the elution profile of PAN and TMX.

The observed chromatographic behaviour of PAN and TMX indicated that the retention of the compounds is based not only on a pure reversed-phase mechanism, but additionally on interactions with the polar silica surface of the column. The strong interaction with the polar silica surface caused irreversible adsorption of the compounds, mainly TMX, on any glass surface of sample containers. In order to avoid these problems, polypropylene tubes and vials were used for preparing, storing and measuring the standard solutions of PAN and I.S. and the biological samples.

Simple and reproducible sample processing is important for a high sample output. Extraction with organic solvents as suggested in the literature [3,4] proved unsuitable as the extracts were found to contain impurities that were poorly separable from PAN.

Sample processing was successful using a protein-precipitation procedure with an equal volume of acetonitrile following solid-phase extraction with a phenyl microcolumn. The deproteinization avoided clogging of the extraction column. A plasma sample deproteinized with acetonitrile was stable even on storage for 4 weeks at −24°C. Addition of an ion-pairing reagent to the acetonitrile-containing plasma improved the absorption of the compounds of interest on the column. Satisfactory elution was achieved only if the microcolumn was adjusted to pH 1 with sulphuric acid before extraction. The procedure resulted in a clean extract with minimal co-extraction of plasma components. The extraction recoveries obtained with the phenyl microcolumns were high and reproducible. The method has proved to be sufficiently sensitive and selective to be used in clinical pharmacokinetic studies.

5. Acknowledgements

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