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DETERMINATION OF TIAPAMIL AND OF ITS TWO MAIN METABOLITES IN PLASMA AND IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Selective high-performance liquid chromatographic methods for the determination of tiapamil and its two main metabolites in plasma and urine are described. Tiapamil together with its metabolites is extracted at alkaline pH into dichloromethane. Separation is carried out using normal-phase high-performance liquid chromatography with ultraviolet detection (278 nm). The unchanged drug and the desmethyl metabolite are analysed simultaneously. The second metabolite is analysed separately under more polar conditions. The sensitivity limits are 50 ng/ml for tiapamil, 100 ng/ml for the desmethyl metabolite and 75 ng/ml for the second metabolite, using 0.5 ml of plasma. The sensitivity limits in urine are 100 ng/ml for all three compounds using a 0.5 ml specimen. The method has been applied to the analysis of human plasma and urine after intravenous (70 mg) and oral (400 mg) administration of tiapamil.

INTRODUCTION

Tiapamil, [I], N-(3,4-dimethoxyphenethyl)-2-(3,4-dimethoxyphenyl)-N-methyl-*m*-dithiane-2-propylamine 1,1,3,3-tetraoxide, is a new calcium antagonist, which is undergoing clinical evaluation as an antiarrhythmic and antihypertensive drug [1, 2]. Tiapamil is extensively metabolized in man [3]. The two main plasma and urine metabolites are the N-desmethyl derivative [II] and another secondary amine [III] (Fig. 1). Both metabolites have low pharmacological activity and do not contribute significantly to the effect of the parent drug.

Tiapamil is structurally related to verapamil. Several analytical procedures have been reported for determining verapamil in plasma, including sensitive

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methods such as gas chromatography—mass spectrometry (GC—MS) and high-performance liquid chromatography (HPLC) with fluorescence detection [4, 5]. However, due to its two sulphone groups, tiapamil cannot be determined readily by GC methods and has only poor fluorescence.

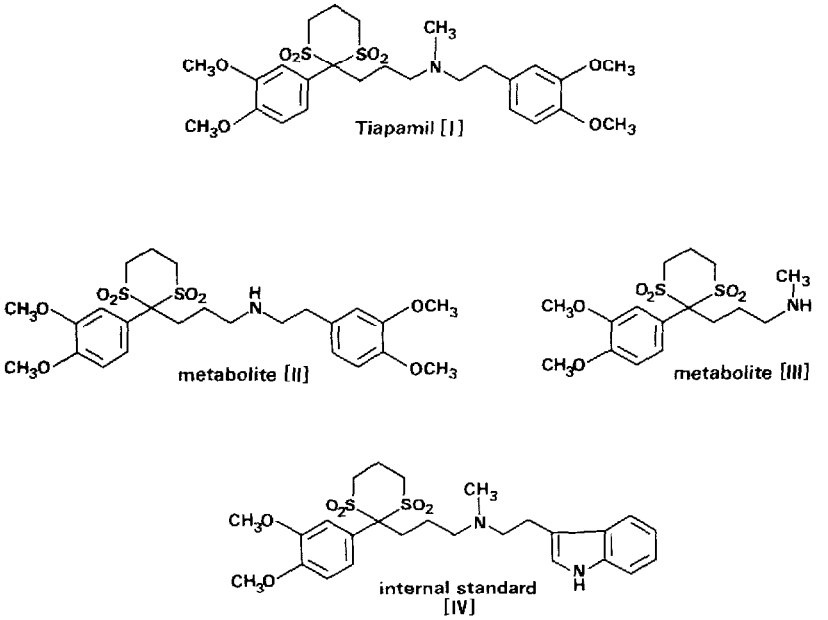

Fig. 1. Chemical structure of tiapamil, its main metabolites and of the internal standard.

In this paper we describe simple and selective normal-phase HPLC methods with ultraviolet (UV) detection for the determination of tiapamil and its two main metabolites in plasma and urine. Tiapamil and metabolite [II] were determined simultaneously. The analogous compound [IV] was used as internal standard. Metabolite [III] was assayed separately by external standardization.

EXPERIMENTAL

Reagents

Chloroform, methanol, dichloromethane, aqueous ammonia (25%), boric acid, and 1 *M* hydrochloric acid were all p.a. grade from E. Merck (F.R.G.). Other reagents were 0.2 mol/l buffer (sodium borate—sodium hydroxide) pH 10, and double-distilled water.

Chromatography

The following modular system was used: Altex Model 110 pump, injector Rheodyne 7125, 10 mV recorder W+W 1100 (all from Kontron Analytik, Switzerland), Knauer UV-detector type 8700 (0.02—0.04 a.u.f.s.) (Knauer, F.R.G.), autosampler ISS-100 (Perkin-Elmer, F.R.G.), computing integrator SP 4100 (Spectra Physics, U.S.A.).

Chromatography of tiapamil and metabolite [II]. A prepacked column (12.5

cm × 4 mm), Hibar RT 125-4, LiChrosorb Si 60, 5 μm, (Merck) was used. The mobile phase was a mixture of chloroform—methanol—25% ammonia (964:35:1) with a flow-rate of 1.2 ml/min at room temperature (90 bars). The monitoring wavelength was 278 nm. Under these conditions, the following retention times (in min) were obtained: t_R [I] = 4.5, t_R [II] = 7.0, internal standard t_R = 10.0.

Chromatography of metabolite [III]. Separations were carried out on a pre-packed column Polygosil 60-5, 12.5 cm × 4 mm (Macherey-Nagel, F.R.G.). The mobile phase was a mixture of chloroform—methanol—25% aqueous ammonia (600:400:1) with a flow-rate of 2 ml/min at room temperature. The monitoring wavelength was 278 nm. Under these conditions the compound eluted at 4.5 min.

Solutions

Tiapamil* (10.64 mg HCl salt, corresponding to 10 mg of free base) was dissolved in 10 ml of water (Solution A).

Metabolite [II]* (11.07 mg fumarate salt, corresponding to 10 mg of free base) was weighed into a 10 ml flask and dissolved in water (6—7 ml) with the aid of sonication. The volume was then made up to 10 ml with water (Solution B).

Metabolite [III]* (10 mg free base) was dissolved in 10 ml of methanol (Solution C).

Internal standard* (10.68 mg HCl salt) was dissolved in 10 ml of methanol. This methanolic solution was then further diluted with water to obtain the concentrations suitable for internal standardization.

Calibration standards

Plasma standards for tiapamil and metabolite [II]. A stock plasma was prepared by adding 200 μl of both Solution A and Solution B (Hamilton syringe) to 50 ml of drug-free plasma (4 μg/ml). This stock plasma was then used to prepare lower concentration standards by stepwise dilution with drug-free plasma. The following standards were prepared: 4 μg/ml (stock plasma), 2, 1, 0.5, 0.25, 0.125 and 0.062 μg/ml.

Plasma standards for metabolite [III]. A stock plasma was prepared by adding 50 μl of Solution C (Hamilton syringe) to 25 ml of drug-free plasma (2 μg/ml). By stepwise dilution of this stock plasma, the following standards were prepared: 2 μg/ml (stock plasma), 1, 0.5, 0.25 and 0.125 μg/ml.

Urine standards for tiapamil and metabolite [II]. A stock urine was prepared by pipetting 5 ml of both Solution A and Solution B into a 50 ml flask and making up to the mark with drug-free urine, resulting in a concentration of 100 μg/ml for both compounds. The following standards were prepared by stepwise dilution with drug-free urine: 100 μg/ml (stock urine), 50, 25, 10, 5, 2.5, 1, 0.5 and 0.25 μg/ml.

Urine standards for metabolite [III]. A stock urine was prepared by adding 100 μl of Solution C to 25 ml of drug-free urine, resulting in a concentration

*Tiapamil (base): Ro 11-1781/00. Metabolite [II] (base): Ro 11-5398/00. Metabolite [III] (base): Ro 11-5220/00. Internal standard: Ro 11-6415.

of 4 μg/ml. By stepwise dilution of this stock urine, the following standards were prepared: 4 μg/ml (stock urine) 2, 1, 0.5, 0.25 and 0.125 μg/ml.

Extraction procedures

Extraction of tiapamil and metabolite [II] from plasma. Plasma (0.5 ml) was pipetted into a conical extraction tube (glass, 15 ml); 200 μl of an aqueous solution of the internal standard and buffer pH 10 (0.5 ml) were then added. After the addition of 10 ml of dichloromethane, the sample was extracted (tumbler extractor, 15 min, 20 rpm) and then centrifuged (1000 *g*) at 10°C for 5 min. The upper aqueous phase was then carefully aspirated and discarded; 9 ml of the organic phase were transferred into a new glass tube and evaporated to dryness under a gentle stream of nitrogen (35°C). The dry residue was redissolved in the eluent (200 μl) and injected for analysis (100 μl).

Extraction of metabolite [III] from plasma. Plasma (0.5 ml) was pipetted into a conical extraction tube. Water (0.5 ml) and buffer pH 10 (1 ml) were added and the mixture was briefly shaken by hand. Following the addition of 10 ml of dichloromethane, the subsequent steps were as described above.

Extraction of tiapamil and metabolite [II] from urine. Urine (0.5 ml) was pipetted into a conical tube, to which an aqueous solution of the internal standard and 0.5 ml of buffer pH 10 were then added. After brief mixing on a Vortex-mixer, 10 ml of dichloromethane were added and the sample was extracted and then centrifuged as described above for plasma samples. The upper aqueous layer was carefully withdrawn by aspiration and discarded; 9 ml of the organic phase were transferred into a new tube; 2 ml of 1 *M* hydrochloric acid solution were added, the tube was rotated for 5 min (20 rpm), followed by centrifugation for 5 min (1000 g, 10°C). The aqueous phase was discarded. An aliquot of the organic phase (taking care to avoid carry-over of the remaining acidic phase) was again transferred into a new tube and evaporated to dryness under a gentle stream of nitrogen. The residue of the extract was immediately redissolved in the eluent (200 μl) and injected for analysis (100 μl).

Extraction metabolite [III] from urine. Urine (0.5 ml) was pipetted into a conical extraction tube. Buffer pH 10 (0.5 ml) was added and the mixture briefly shaken by hand. The subsequent steps were as described for plasma samples.

Calibration

Standards (4—5) covering the concentration ranges described below, were processed as described above and analysed alongside the unknown samples. For the determination of tiapamil and [II], the peak height ratios of unchanged drug to the internal standard were measured and the calibration curve was obtained from the least-squares linear regression of the peak height ratio against concentration. This regression line was then used to calculate the concentration of the unchanged drug in unknown samples. Calibration ranges for [I] and [II] were as follows: 0.06—0.5 μg/ml and 0.25—4 μg/ml for plasma; 0.25—5 μg/ml and 5—100 μg/ml for urine. For the determination of [III], external standardization was used. Calibration ranges were 0.125—2 μg/ml (plasma) and 0.125—4 μg/ml (urine).

RESULTS

Recovery

Spiked plasma and urine samples of various concentrations were prepared and extracted as described above, except that the internal standard was not added. Another series of standards was prepared by adding solutions of the free bases of tiapamil, [II] and [III] in the eluent to extracts of drug-free plasma or urine. The peak heights obtained from this latter experiment provided the 100% values, which could be compared with the peak heights obtained from the extracted spiked standards. The mean recoveries were as follows: tiapamil, plasma 72%, urine 101%; metabolite [II], plasma 79%, urine 90%; metabolite [III], plasma 99%, urine 95%.

Linearity

Linear correlations between peak height ratios and the concentrations of tiapamil and [II] (respectively between peak height and the concentration of [III]) were found in the range of the above-mentioned calibration standards.

Reproducibility

Inter-assay reproducibility was calculated from spiked plasma and urine samples of various concentrations, which were analysed as replicates on different days, using a new calibration each day. Tables I and II present the

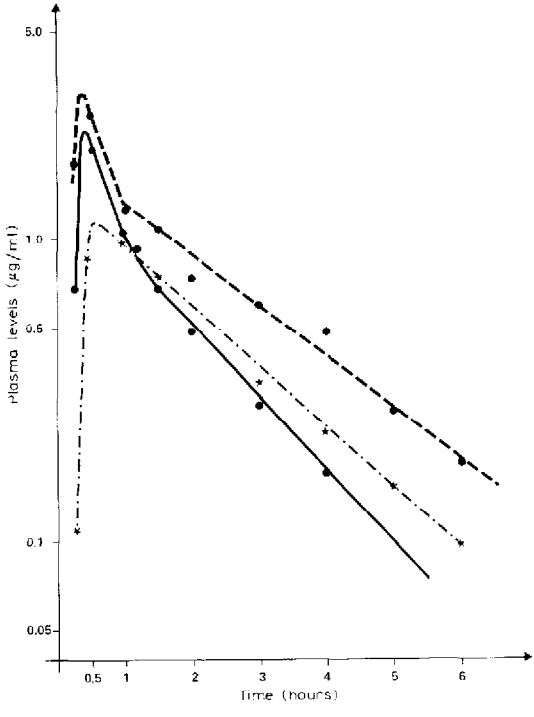

Fig. 2. Plasma levels of tiapamil and metabolites after a single oral administration of 400 mg of tiapamil to a healthy volunteer. (— — —), tiapamil; (——), metabolite [II]; (— • — •), metabolite [III].

TABLE I

INTER-ASSAY REPRODUCIBILITY (C.V.) AND ACCURACY OF THE PLASMA ASSAY

Conc. added (μg/ml)	[I]			[II]			[III]			*n*
	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	
0.12	0.12	13	± 0	0.13	12	+ 8.3				7
0.31	0.32	5.6	+ 3.2	0.33	4.3	+ 6.0				12
0.65	0.61	4.6	− 6.1	0.66	5.7	+ 1.5				6
2.50	2.60	4.5	+ 4.0	2.60	6.2	+ 4.0				11
0.10							0.10	16	± 0	4
0.25							0.26	4.8	+ 4.0	4
0.50							0.51	7.5	+ 2.0	5
1.00							0.99	2.6	− 2.6	5

TABLE II

INTER-ASSAY REPRODUCIBILITY (C.V.) AND ACCURACY OF THE URINE ASSAY

Conc. added (μg/ml)	[I]			[II]			[III]			*n*
	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	
0.25	0.26	10	+ 4	0.25	2.8	± 0				7
1.25	1.23	3.0	− 1.6	1.24	3.0	− 0.8				6
5.0	4.82	2.6	− 3.6	5.23	3.4	+ 4.6				3
25.0	23.6	5.0	− 5.6	24.3	5.0	− 2.8				6
100	96.2	0.5	− 3.8	98.0	1.8	− 2.0				3
0.25							0.24	11.3	− 4.0	4
1.0							1.00	2.6	± 0	5
25.0							24.5	5.4	− 2.0	7
100							98.4	7.2	− 1.6	5

TABLE III

CONTENT OF TIAPAMIL AND OF ITS METABOLITES IN URINE AFTER A SINGLE INTRAVENOUS DOSE OF 70 mg OF TIAPAMIL TO A HEALTHY VOLUNTEER

Time after administration (h)	Urine volume (ml)	Concentration (μg/ml)			Cumulative amount excreted (mg)		
		Tiapamil [I]	[II]	[III]	Tiapamil [I]	[II]	[III]
0—8	1300	11.20	0.30	3.0	14.56	0.39	3.90
8—24	850	1.23	n.d.*	1.55	15.61	0.39	5.05
24—32	525	0.24	n.d.	0.49	15.74	0.39	5.31
32—48	900	n.d.	n.d.	0.21	15.74	0.39	5.50

*n.d. = below limit of detection.

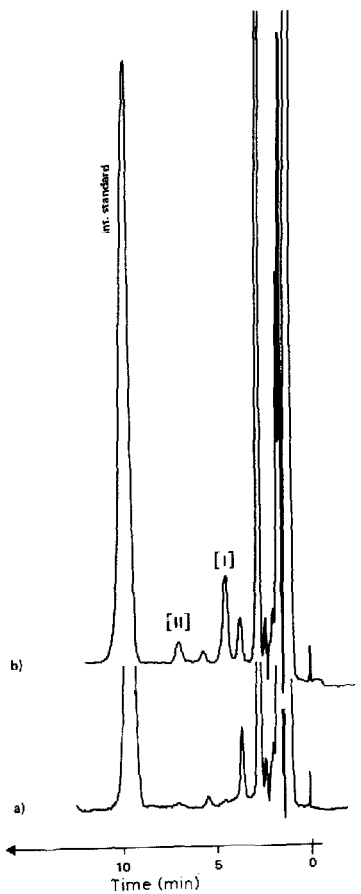

Fig. 3. Determination of tiapamil and [II] in plasma. Chromatograms of plasma extracts of a volunteer having received an intravenous dose of tiapamil (1 mg/kg). Detector range setting: 0.02 a.u.f.s. (a) drug-free plasma; (b) plasma, 45 min after administration. Tiapamil [I] = 210 ng/ml; metabolite [II] = 100 ng/ml.

inter-assay reproducibility (C.V., %) and accuracy (difference between found and added concentration) at different concentrations. The overall precision of the plasma and of the urine assay was about 5%. In the low concentration range (⩽ twice the limit of detection) the coefficient of variation was about 10%. Accuracy was better than two times the standard deviation (S.D.).

Limit of detection

Using 0.5 ml of plasma, the limits of detection were 50 ng/ml for tiapamil, 100 ng/ml for [II] and 75 ng/ml for [III]. For 50 ng/ml tiapamil a 5 mm peak height was obtained, the signal-to-noise ratio being 6:1. Using 1 ml of plasma, a lower detection limit could possibly be obtained. For urine samples, the limit of detection was found to be 100 ng/ml for all three compounds.

Stability of the drug in plasma

Tiapamil and its metabolites were found to be stable in plasma at −18° C for at least six months.

Application of the method to biological samples

The method has been applied to the analysis of tiapamil and its main metabolites in plasma and urine of volunteers and patients after intravenous and oral

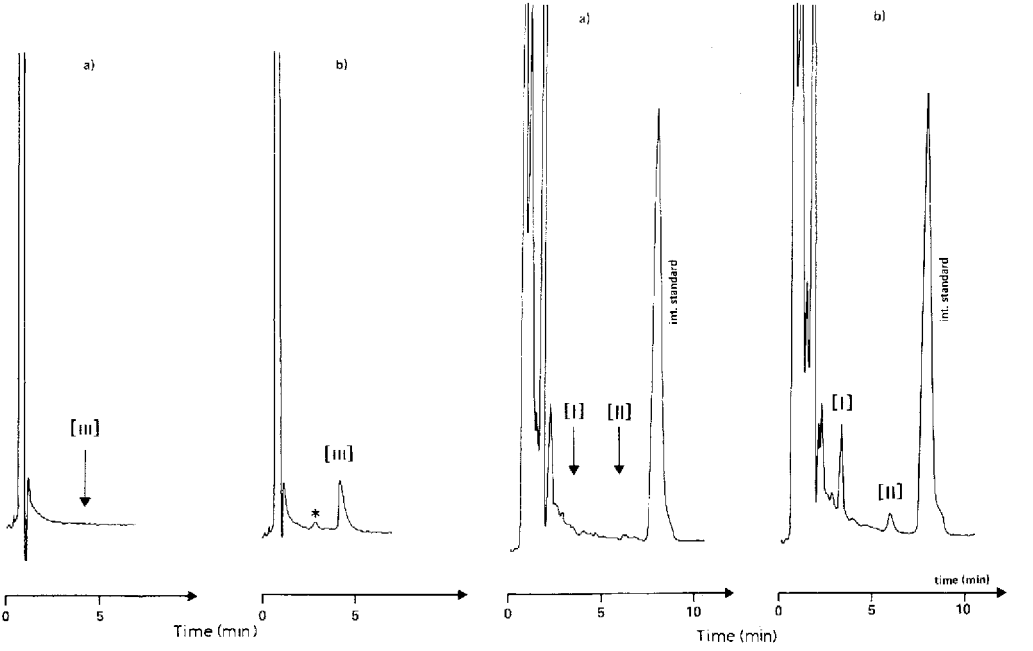

Fig. 4. Determination of [III] in plasma. Chromatograms of plasma extracts of a volunteer having received an intravenous dose of tiapamil (70 mg). Detector range setting: 0.02 a.u.f.s. (a) pre-dose sample, (b) plasma, 40 min after administration. Metabolite [III] = 240 ng/ml; * = minor metabolite.

Fig. 5. Determination of tiapamil and [II] in urine. Chromatograms of urine extracts of a volunteer having received an intravenous dose of tiapamil (70 mg). Detector range setting: 0.02 a.u.f.s.; flow-rate 2.3 ml/min. (a) Pre-dose urine; (b) collection period 24—32 h after administration. Tiapamil [I] = 270 ng/ml; metabolite [II] = 150 ng/ml.

administration. Plasma concentrations of tiapamil and of its metabolites after an oral dose of 400 mg are indicated in Fig. 2. Urine concentrations after an oral dose of 400 mg are shown in Table III. Chromatograms of plasma and urine extracts from volunteers having received a 70 mg intravenous dose are shown in Figs. 3—5.

DISCUSSION

Tiapamil, together with its two metabolites, is extracted at alkaline pH into dichloromethane. However, as the polarity of [III] is rather different from that of the two other compounds, a simultaneous determination of these three compounds could not be achieved under isocratic conditions. Therefore, [III] had to be analysed separately under more polar conditions.

The chromatogram illustrated by Fig. 3 represents the normal separation of tiapamil from endogenous peaks, that could be obtained in routine analysis. In the case of less efficient columns or in the presence of interfering comedication, adequate separation could be achieved by reducing the methanol and/or ammonia content of the mobile phase (for instance, to 974:25:1 chloroform—methanol—ammonia).

The eluent proportions 964:35:1 were also used for the analysis of urine samples from healthy volunteers. For the analysis of urine samples from patients, it was sometimes advantageous to reduce the content of methanol or ammonia. In this way, the separation from comedicated drugs could be improved.

For the determination of unchanged drug and [II] in urine, especially in the low concentration range (below 250 ng/ml), it was necessary in most cases to wash the organic extract with 1 *M* hydrochloric acid. Tiapamil and [II] remained in the organic phase, whereas interfering compounds and [III] were extracted into the acidic phase. Some of the urine samples, especially in the higher concentration range, could be analysed without this clean-up step.

In addition, this clean-up was sometimes necessary for the analysis of plasma samples from patients.

The recovery for tiapamil and [II] was lower for plasma samples than for urine samples, whereas the recovery of [III] was not affected by the change in sample matrix. This result may be explained by strong binding of these two compounds to protein materials in the plasma.

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