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Note

Ion-exchange high-performance liquid chromatography in drug assay in biological fluids

II. Verapamil

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In continuation of our study of the potentialities of ion-exchange sorbents in high-performance liquid chromatography (HPLC) of drugs in biological fluids [1], we applied such sorbents for the determination of verapamil in serum, saliva and urine. Verapamil hydrochloride [2,8-bis(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile hydrochloride] is a potent cardiovascular drug with antianginal and antiarrhythmic activity [2].

Current methods for analysing verapamil in biological fluids are based on gas—liquid chromatography [3—6] and HPLC with reversed-phase columns [7—9]. Two of them [3,9] are able to determine also the N-desmethylated metabolite of verapamil (norverapamil) which is pharmacologically active [2]. The method presented here allows good separation of unchanged verapamil and its three main metabolites: norverapamil, 2-(3,4-dimethoxyphenyl)-2-isopropyl-6-azaheptanitrile (D 617) and 2-(3,4-dimethoxyphenyl)-2-propylamino-3-methylbutyronitrile (D 620).

While this work was in progress two papers appeared concerning HPLC determination of verapamil. One of them [10] described ion-paired reversed-phase separation of verapamil and its seven metabolites, but did not allow norverapamil determination. Cole et al. [11] separated the same compounds as we did using adsorption column, but their method was suitable only for plasma or serum. It is necessary also to note that potassium bromide, which was used by Cole et al. as a mobile phase component, is not recommended along with other halide salts by manufacturers of commonly used liquid chromatographic systems.

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EXPERIMENTAL

Apparatus and columns

The chromatographic system consisted of a Model 110 pump (Altex, Berkeley, CA, U.S.A.), a Model 7120 injection valve (Rheodyne, Berkeley, CA, U.S.A.) and a Model FS-970 fluorescence detector (Schoeffel, Westwood, NY, U.S.A.). The separation was carried out on a 25 cm × 4.6 mm I.D. Partisil 10 SCX column (Altex), with a precolumn of 4 cm × 3.2 mm I.D. packed by us with the same sorbent. Recorder Model 355 (Linear Instruments, Irvine, CA, U.S.A.) was used.

Reagents and standards

Pentane and amyl alcohol were of pure grade and were distilled in glass prior to use. Diethylamine was of pure grade and was distilled over sodium hydroxide. Acetonitrile (LiChrosolv®, E. Merck, Darmstadt, G.F.R.) was used as received. Other reagents were of analytical grade or of chemically pure grade. The water used for preparation of solutions and the mobile phase was double distilled in glass.

Verapamil hydrochloride (LEK, Ljubljana, Yugoslavia) and glaucin hydrochloride (Tatchimpharmpreparaty, Kasan, U.S.S.R.) were used for standard solution preparations. Three concentrations were prepared: 100, 10 and 1 μg/ml.

Extraction procedure

Samples of serum (1 ml), saliva (1 ml) or urine (0.1—1.0 ml aliquot) were pipetted into 15-ml glass-stoppered Pyrex glass tubes (silanised before assay). In the case of urine the volume was adjusted to 1 ml with distilled water. Then 50—100 μl of the internal standard solution (glaucin) of the appropriate concentration were added followed by 100 μl of 0.1 *N* potassium hydroxide solution. Samples were extracted for 30 sec with 5 ml of pentane—amyl alcohol (20:1) using a Vortex mixer. Layers were separated by centrifugation for 5 min at 500 *g*; the organic layer was transferred into the conical tube and extraction was repeated with 2 ml of the same mixture for 15 sec. The combined extract was vortexed with 100 μl of 0.1 *N* sulphuric acid. After a short centrifugation the acidic extract (50—90 μl) was injected into the column.

Chromatographic conditions

The mobile phase was prepared by mixing acetonitrile (200 ml), diethylamine (0.86 ml), glacial acetic acid (0.57 ml) and water (up to 1000 ml). The mixture was degassed under vacuum. The flow-rate was 2 ml/min, the column temperature was ambient.

The excitation wavelength was 206 nm, the emission filter was not used. Sensitivity was set at 3.5; time constant was 0.5 sec.

Quantitation

The internal standard method was used for verapamil determination using glaucin (4,5,7,8-tetramethylaporphine hydrochloride) as the internal standard. Calibration curves were obtained by assaying the samples spiked with verapamil

in the concentration range 10—500 ng/ml (10—1000 ng/ml for urine). Peak height ratios were plotted against verapamil concentrations. Recovery was assessed by comparing peak heights after analysis of extracted drugs and standard solutions.

Mass spectrometry

The peaks were collected in conical tubes and the mobile phase was evaporated by rotary evaporator at 60°C. Residue was reconstituted in methanol. The mass spectra were obtained on a Varian MAT-112 mass spectrometer at 50 eV with direct injection of samples.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms of extracts of blank serum (a) and serum spiked with verapamil and internal standard (glaucin), 20 and 10 ng/ml, respectively (b). In Fig. 2a the chromatogram of urine free of drugs is presented. As one can see, there are no peaks on the chromatograms which interfere with verapamil and glaucin. The same results were obtained with saliva. Fig. 1c and Fig. 2b show, respectively, chromatograms of extracts of serum and urine taken from a patient who received verapamil orally. Peak 5 represents glaucin, and peak 4 is intact verapamil; this was confirmed by the mass spectrum, which fully coincides with one published [6]: *m/e* 454 (M^+, relative intensity 0.5%), 305 (3%), 304 (29%), 303 (100%), 261 (1%), 260 (5%). Peak 3 was identified as norverapamil: *m/e* 440 (M^+, 0.5%), 439 (0.7%), 290 (24%), 289 (100%), 260 (10%); this also corresponded to published data [6]. Peak 2 represents D 617, the product of N-dealkylation of verapamil: *m/e* 291 (1%), 290 (M^+, 3%), 259 (1%), 247 (3%), 219 (1%), 216 (2%). The peak of maximum intensity had *m/e* 44, which coincided with previous data [6]. The mass spectrum of peak 1 is presented in Fig. 3. The *m/e* values of peaks in it correspond to the fragmentation scheme of D 620, but relative intensities differ from those presented previously [6]. We suppose, however, that peak 1 corresponded exactly to the product of verapamil N-dealkylation because the rest of the verapamil metabolites had phenolic hydroxy groups and could not be extracted from alkaline medium.

Thus, our method gives the possibility to determine simultaneously intact verapamil and its three main metabolites. As we did not have the metabolite standards at our disposal we only quantitated intact verapamil.

In Table I regression equations of the calibration curves for the determination of verapamil in serum, saliva and urine are presented. Because the therapeutic levels of verapamil do not exceed 500 ng/ml [2], the calibration curves for serum and saliva were constructed in the range 10—500 ng/ml. For urine the range 10—1000 ng/ml was chosen. The correlation coefficients for the regression lines given in Table I represent the excellent linearity of the calibration curves.

The recovery was determined at two levels of verapamil and glaucin in serum, saliva and urine, and the results are given in Table II. As one can see, the recovery of verapamil exceeds 65% and that of glaucin 70%.

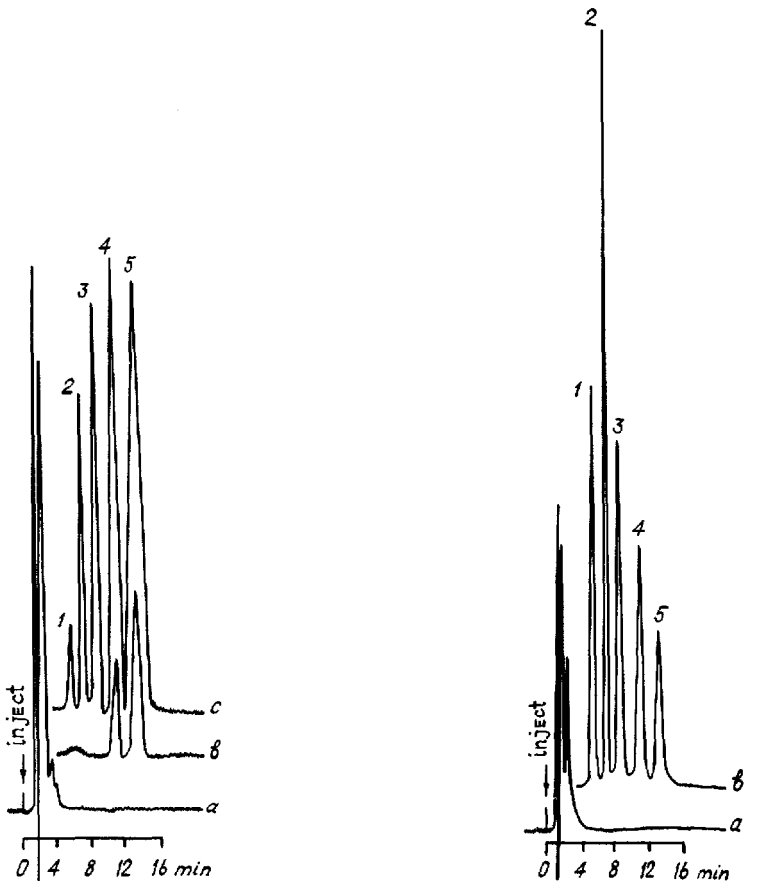

Fig. 1. (a) Chromatogram of the extract of a blank serum. (b) Chromatogram of the extract of the same serum spiked with 20 ng of verapamil and 10 ng of glaucin (the scale range is 0.02 μA). (c) Chromatogram of the serum extract of a patient who received verapamil orally. Peaks: 1 = D 620, 2 = D 617, 3 = norverapamil, 4 = verapamil, 5 = internal standard (glaucin).

Fig. 2. (a) Chromatogram of the extract of a blank urine. (b) Chromatogram of the urine extract of a patient who received verapamil orally. Peak identification as in Fig. 1.

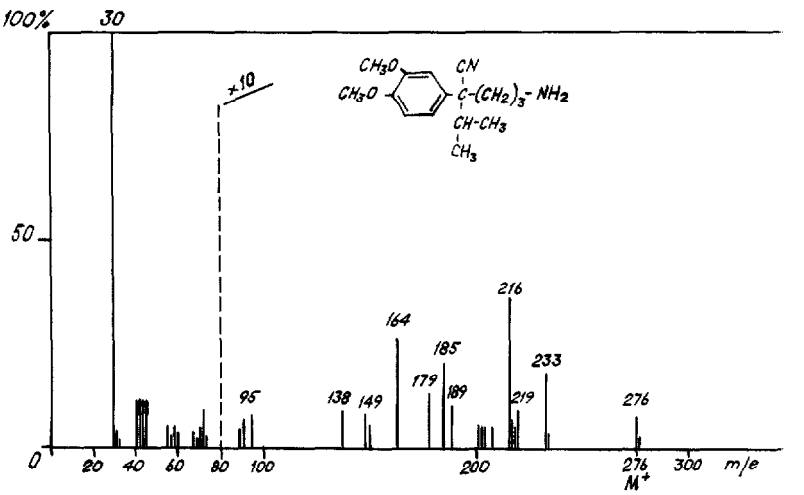

Fig. 3. Mass spectrum of metabolite 1. For mass spectrometric conditions see text.

TABLE I

REGRESSION EQUATIONS OF THE CALIBRATION CURVES FOR VERAPAMIL ASSAY IN SERUM, SALIVA AND URINE

Biological fluid	Regression equation*	Correlation coefficient
Serum	$y = 0.0030x + 0.0102$	0.995
Saliva	$y = 0.0031x + 0.0165$	0.995
Urine	$y = 0.0038x + 0.0313$	0.990

*x = verapamil concentration; y = verapamil to glaucin peak height ratio.

TABLE II

EXTRACTION YIELD OF VERAPAMIL AND GLAUCIN FROM SERUM, SALIVA AND URINE

Concentration added (ng/ml)		n	Recovery (%)	Coefficient of variation (%)
Serum				
Verapamil	20	10	67.8 ± 1.9	9.1
	100	5	71.7 ± 2.8	8.6
Glaucin	50	5	78.2 ± 2.7	7.7
	100	5	80.1 ± 2.4	7.5
Saliva				
Verapamil	20	5	66.5 ± 3.8	11.9
	100	7	66.7 ± 1.5	5.8
Glaucin	50	7	80.4 ± 2.3	7.7
	100	5	80.7 ± 2.2	6.5
Urine				
Verapamil	50	5	80.5 ± 4.2	11.5
	500	6	85.4 ± 1.4	4.2
Glaucin	100	5	69.5 ± 1.3	4.2
	500	5	72.2 ± 1.2	4.0

TABLE III

ACCURACY OF THE DETERMINATION (day-to-day) OF VERAPAMIL IN SERUM, SALIVA AND URINE

$n = 5$.

Concentration of verapamil (ng/ml)		Coefficient of variation (%)
Serum	20	5.5
	100	4.9
Saliva	20	7.7
	100	6.2
Urine	50	10.3
	500	9.0

TABLE IV.

DRUGS WHICH DO NOT INTERFERE IN VERAPAMIL DETERMINATION

Amitriptyline	Isosorbide dinitrate
Amphetamine*	Lidocaine
Amylobarbital	Mexiletine
Aspirin	Nadolol*
Barbital	Nitroglycerin
Butabarbital	Oxazepam
Diazepam	Papaverine
Digoxin	Phenobarbital
Dilantin	Prazosin*
Ephedrine	Propranolol*
Etmosin	Reserpine
Furosemide	Secobarbital
Hypothiazide	Trimecaine

*These drugs interfered with metabolite peaks.

In Table III the data on the accuracy of the varapamil determination are presented. The detection limit of the method is about 5 ng/ml (peak-to-noise ratio 5:1) which is sufficient for the pharmacokinetic study of verapamil.

In Table IV the drugs which do not interfere in the determination of intact verapamil are listed. Certain drugs interfered with the metabolite peaks.

In conclusion, a specific and sensitive method has been developed for verapamil assay in serum, saliva and urine using a cation-exchange Partisil 10 SCX column and fluorescence detection, which has some advantages over published methods. The extraction procedure is not time-consuming because the stage of solvent evaporation is excluded. During a day 30 samples may be worked-up. The acid reextract can be stored at 4°C for several days without any change. The chromatographic analysis of one sample takes no more than 16 min.

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