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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CICLETANIDE, A NEW DIURETIC, IN PLASMA, RED BLOOD CELLS, URINE AND SALIVA

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SUMMARY

A sensitive, selective and easy to use high-performance liquid chromatographic method for the determination of cicletanide, a new diuretic, in plasma, red blood cells, urine and saliva is described. After extraction of cicletanide together with an internal standard with diethyl ether, or diethyl ether—*n*-hexane (20:80) for urine, the sample extracts are chromatographed with water—methanol—acetic acid (50:50:0.3) as eluent on to a Nucleosil C_{18} column. Both compounds are detected by their ultraviolet absorption at 280 nm. The calibration graph was linear between 0.2 and 20 μg/ml for plasma and between 0.2 and 5 μg/ml for the other biological fluids. The sensitivity limit was 20 ng/ml for plasma, red blood cells and saliva and 30 ng/ml for urine. The coefficients of variation of the between-day assays did not exceed 4.6% in plasma, 8.3% in red blood cells, 7.8% in urine and 4.2% in saliva for the lowest concentrations studied. The application of the method to a pharmacokinetic study of cicletanide after a single oral therapeutic dose in humans is reported.

INTRODUCTION

Cicletanide [2-methyl-3-hydroxy-4*H*,5*H*-5-(4′-chlorophenyl)isofuropyridine hydrochloride] (PXD) is a new compound (IPSEN Labs.) that exhibits marked diuretic and anti-hypertensive properties [1]. Owing to these pharmacological properties, PXD is intended for use in the treatment of hypertension, a disease that is frequently associated with renal impairment. Therefore, the influence of renal insufficiency on the pharmacokinetics of PXD should be studied. Such a study can only be conducted with actual patients, so it requires a highly selective analytical technique with a sufficiently high sensitivity to allow the

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measurement of the drug concentration after the administration of only one therapeutic dose.

Until now, only one method has been described [2] for the determination of PXD in plasma and urine and it exhibits some drawbacks such as the lack of an internal standard for urine and a relatively poor reproducibility. As a consequence, it appeared of major importance to devise a technique that would allow the precise measurement of PXD in plasma and urine and also in red blood cells and saliva in order to obtain a larger amount of information concerning the kinetics and the disposition of the drug. In this paper, an easy to use high-performance liquid chromatographic technique is described and its application to the kinetic study of a single oral therapeutic dose of PXD in subjects with normal renal function is demonstrated.

EXPERIMENTAL

Standards and reagents

PXD and 2-methyl-3-hydroxy-4*H*-5-methyl-5-(4′-chlorophenyl)isofuropyridine hydrochloride, used as an internal standard (I.S.), were supplied by IPSEN Labs. (Paris, France).

Diethyl ether (Carlo Erba, Milan, Italy) and methanol (Prolabo, Paris, France) were distilled before use. *n*-Hexane (Merck, Darmstadt, F.R.G.) and acetic acid were of analytical-reagent grade. Diethyl ether was used as the extraction solvent for plasma, red blood cells and saliva and diethyl ether—*n*-hexane (20:80) was used for urine.

Stock solutions (100 μg/ml) of PXD and the internal standard were prepared in a mixture of methanol and twice-distilled water (1:9) and were found to be stable at 4°C for at least six months. Working solutions were prepared twice a month by diluting the stock solutions with the same solvent.

Biological fluids

Human blood obtained from the local blood bank was centrifuged at 4°C and 5-ml aliquots of separated plasma were stored at −20°C, while the remaining red blood cells were washed twice with sterile saline, the volume of which was equal to that of the discarded plasma. After the last centrifugation (600 *g*) and removal of sterile saline, 5-ml aliquots of haemolysate of red blood cells were obtained by freezing and then stored at −20°C. A human urine pool was collected from male and female volunteers and 10-ml aliquots were stored at −20°C. Human saliva was collected from a male volunteer shortly before analysis.

Apparatus and chromatographic conditions

Analyses were performed by using a chromatographic system that consisted of a Model 410 high-pressure pump (Kontron, Zurich, Switzerland), equipped with a Model 810 pulse damper and a Model 7125 100-μl loop injector (Rheodyne, Cotati, CA, U.S.A.). A Uvikon 720 LC variable-wavelength detector (Kontron) (2 nm slits) allowed the detection of both compounds at 280 nm. The stainless-steel column (25 cm × 4.6 mm I.D.) was packed with Nucleosil C_{18}, particle size 10 μm (Macherey, Nagel & Co., Düren, F.R.G.).

The mobile phase was a mixture of methanol and 0.1 *M* acetic acid in twice-distilled water (50:50), which was delivered at a flow-rate of 1 ml/min, producing a pressure of 75 bars at 22°C (air conditioning).

Procedure

Into a glass-stoppered centrifuge tube, 1 ml of internal standard solution (10 μg/ml), 1 ml of biological sample and 7 ml of extraction solvent were successively introduced. The mixture was shaken for 15 min on a rotating mixer (60 rpm) and then centrifuged (1800 *g*) for 15 min at 4°C. A 5-ml volume of the organic layer was transferred into a conical test-tube. This extraction step was applied again to the biological residue, then 5 ml of solvent were introduced and a further 5 ml of the organic layer was added to the previous one. The solvent was evaporated to dryness under a gentle stream of nitrogen in a water-bath at 37°C. Subsequently 0.25—0.5 ml of methanol—twice-distilled water (1:9) were added to the dry residue 30 min prior to injection and 100 μl of this solution were chromatographed.

RESULTS

Chromatographic data

Typical chromatograms obtained for blank plasma, red blood cells, urine and saliva before and after spiking with known amounts of PXD and for biological samples from a patient having received 300 mg of PXD 3 h before sampling, are shown in Fig. 1. The most important chromatographic characteristics obtained are given in Table I. The high value obtained for the resolution factor (2.6) between PXD and the internal standard with k' values of 2.9 and 4.4, respectively, and with an analysis time of less than 15 min are noteworthy. According to the low asymmetry factors (1.1 and 1.35 for PXD and the internal standard, respectively) both peak heights and peak areas could be used for the calculations.

In addition, it should be noted that no interference from endogenous substances were observed. However, for urine such a result could be obtained only when using diethyl ether—*n*-hexane (20:80) as the extraction solvent.

Linearity

For each biological fluid a calibration graph was generated by spiking samples of the corresponding fluid with various amounts of PXD (concentration range 0.2—20 μg/ml for plasma and 0.2—5 μg/ml for urine, red blood cells and saliva) and analysing them by the method already described. In each instance a linearity test was applied to the data obtained from six assays for each concentration studied and a linear relationship was found between the peak-height ratio of PXD to the internal standard and the PXD concentration. The results (Table II) indicate a high value of the regression coefficients associated with a low value of the intercept, which confirms the lack of endogenous interference, as already shown in Fig. 1. The slopes of the regression lines were similar for the various fluids tested, except for urine, owing to the different extraction solvent used.

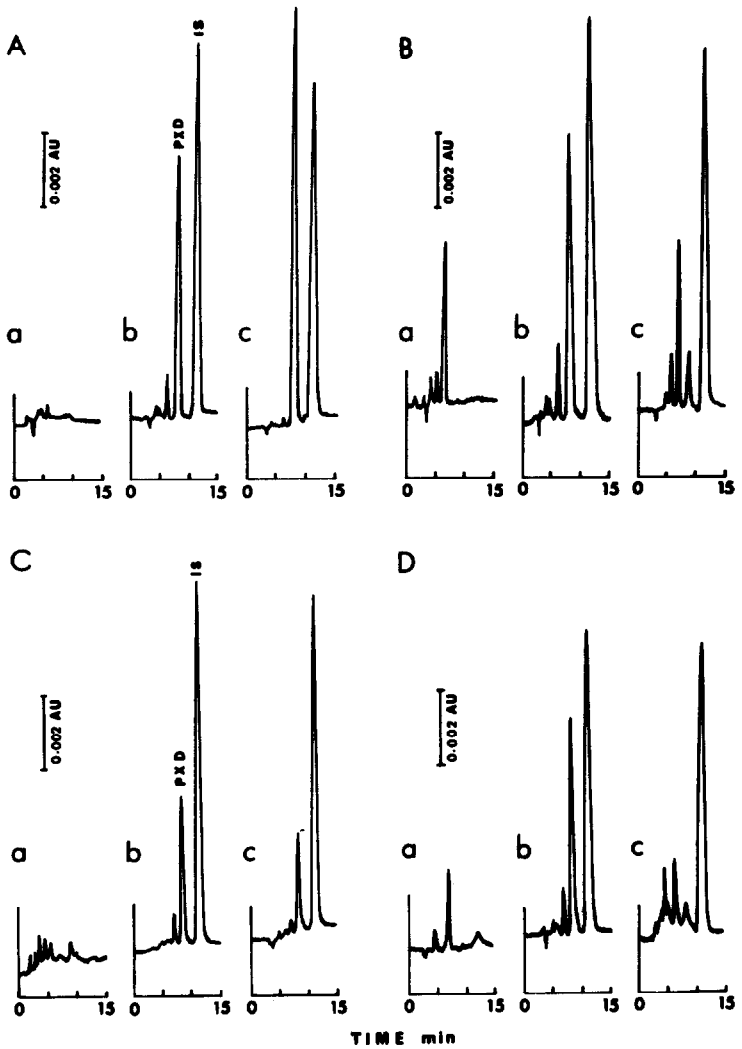

Fig. 1. Chromatograms obtained from extracts of (A) plasma, (B) red blood cells, (C) urine and (D) saliva. (a) Controls; (b) samples spiked with PXD (5 μg/ml) and I.S. (10 μg/ml); (c) samples from a subject having received a single oral dose of 300 mg of PXD 3 h before sampling.

TABLE I

CHROMATOGRAPHIC PARAMETERS FROM PXD AND THE INTERNAL STANDARD

Compound	Retention time (min)	k'	Resolution factor	Asymmetry factor
PXD	7.8	2.9	2.6	1.10
I.S.	10.8	4.4		1.35

TABLE II

PARAMETERS OF LINEAR CALIBRATION GRAPHS

n = 6 for all determinations.

Biological fluid	PXD concentration range (μg/ml)	Slope	S.D. of slope	Intercept	S.D. of intercept	Regression coefficient, r^2
Plasma	0.2—20	0.1314	0.0028	0.0063	0.0029	0.9996
Red blood cells	0.2— 5	0.1317	0.0056	—0.0005	0.0001	0.9982
Urine	0.2— 5	0.0875	0.0028	0.0069	0.0025	0.9990
Saliva	0.2— 5	0.1353	0.0014	—0.0071	0.0024	0.9988

Recovery

The overall recovery was determined by comparing the peak heights of PXD and the internal standard obtained after injection of standard solutions with those obtained after injection of solutions reconstituted from extracts of spiked biological samples. The results in Table III show that for plasma, red blood cells and saliva the recoveries of PXD are similar to those of the internal standard (around 78%), with low coefficients of variation. In contrast, for urine, owing to the change of extraction solvent, the recoveries of PXD and the internal standard were lower and higher, respectively, than those observed for the other fluids.

TABLE III

RECOVERY OF PXD AND OF THE INTERNAL STANDARD IN PLASMA, RED BLOOD CELLS, URINE AND SALIVA

n = 5 for all determinations.

Biological fluid	PXD concentration range (μg/ml)	Recovery (%)	C.V. (%)	I.S. concentration (μg/ml)	Recovery (%)	C.V. (%)
Plasma	0.2—20	81.5	2.1	10	73.8	1.9
Red blood cells	0.2— 5	79.1	2.4	10	75.2	2.3
Urine	0.2— 5	67.2	3.7	10	93.4	2.8
Saliva	0.2— 5	80.2	2.5	10	76.1	2.2

Sensitivity and precision

Under the experimental conditions described above, the sensitivity limit was 20 ng/ml for plasma, red blood cells and saliva and 30 ng/ml for urine. The assay sensitivity might be further increased by using 2 ml of biological fluid without modifying the procedure.

The precision of the assay was established by replicate analyses of samples over the concentration range defined for the study of linearity, which represents the entire range of PXD levels currently encountered after a single oral therapeutic dose in humans. For all the biological fluids studied, the within-day precision determined on six spiked PXD samples of each fluid was

TABLE IV

BETWEEN-DAY PRECISION OF PXD MEASUREMENT IN SPIKED PLASMA, RED BLOOD CELLS, URINE AND SALIVA

n = 6 for all determinations.

Biological fluid	Amount of PXD added (μg/ml)	Amoutn of PXD found (μg/ml) (mean ± S.D.)	C.V. (%)
Plasma	0.2	0.21 ± 0.01	4.6
	0.5	0.49 ± 0.01	2.3
	1	1.00 ± 0.01	1.3
	2	2.00 ± 0.03	1.6
	5	5.00 ± 0.10	2.0
	10	10.63 ± 0.28	2.6
	20	20.22 ± 0.17	0.8
Red blood cells	0.2	0.24 ± 0.02	8.3
	0.5	0.52 ± 0.02	3.8
	1	1.00 ± 0.04	4.0
	2	1.90 ± 0.07	3.7
	5	5.00 ± 0.02	4.0
Urine	0.2	0.18 ± 0.01	5.5
	0.5	0.51 ± 0.04	7.8
	1	1.00 ± 0.04	4.0
	2	2.00 ± 0.07	3.5
	5	5.00 ± 0.16	3.2
Saliva	0.2	0.24 ± 0.01	4.2
	0.5	0.53 ± 0.01	1.8
	1	1.00 ± 0.03	3.1
	2	1.90 ± 0.06	3.1
	5	5.00 ± 0.07	1.4

2.4 and 2.5% for concentrations of 5 and 0.5 μg/ml, respectively. The between-day precision is shown in Table IV. From these results, it appeared that the coefficients of variation did not exceed 4.6% in plasma, 8.3% in red blood cells, 7.8% in urine and 4.2% in saliva for the lowest concentrations studied.

DISCUSSION

As stated in the Introduction, previously only one method for determining PXD has been described [2] and it did not meet all the requirements for a precise pharmacokinetic study, especially for the determination of urinary excretion. The technique described here is simple and rapid, and can be applied to measure PXD accurately in four biological fluids.

Although PXD and the internal standard have very low solubilities in water, it must be emphasized that owing to their reversed-phase chromatographic behaviour both compounds must be injected as partly aqueous solutions (methanol—water) in order to obtain profiles with non-tailing peaks.

Although interferences from endogenous substances were easily avoided for

plasma, red blood cells and saliva, for urine this could only be achieved by using diethyl ether—*n*-hexane instead of pure diethyl ether as extraction solvent. This modification was necessary in order to reduce the front solvent peak so as to be able to determine amounts of PXD smaller than 0.5 μg/ml. For this purpose a proportion of 80% of *n*-hexane in the mixed solvent was required in order to remove the largest part of the interfering endogenous compounds within the limits of the extraction capacity of the solvent for PXD and the internal standard. In addition, the possible interference of several drugs that could be administered together with PXD, such as other diuretics (furosemide, hydrochlorothiazide), β-blockers (propranolol, atenolol), analgesics (aspirin, paracetamol) and tranquillizers (diazepam, lorazepam, oxazepam, nitrazepam) was tested by using samples drawn from patients treated with these drugs. Most of these compounds and their metabolites were not detectable under the chromatographic conditions used and the others were not extracted by the solvents used.

The reliability of the method was evaluated by carrying out fifty different analyses of two spiked samples used as controls with every batch of samples to be analysed over a six-months period. The stability of the chromatographic system was excellent as no change could be observed within this routine work period insofar, column clean-up being performed each forty to fifty assays. This was achieved by using methanol at a flow-rate of 1.5 ml/min for 1 h, after which the stability of the column was ensured by passing the mobile phase for 30 min.

The method was devised for pharmacokinetic studies and as a typical

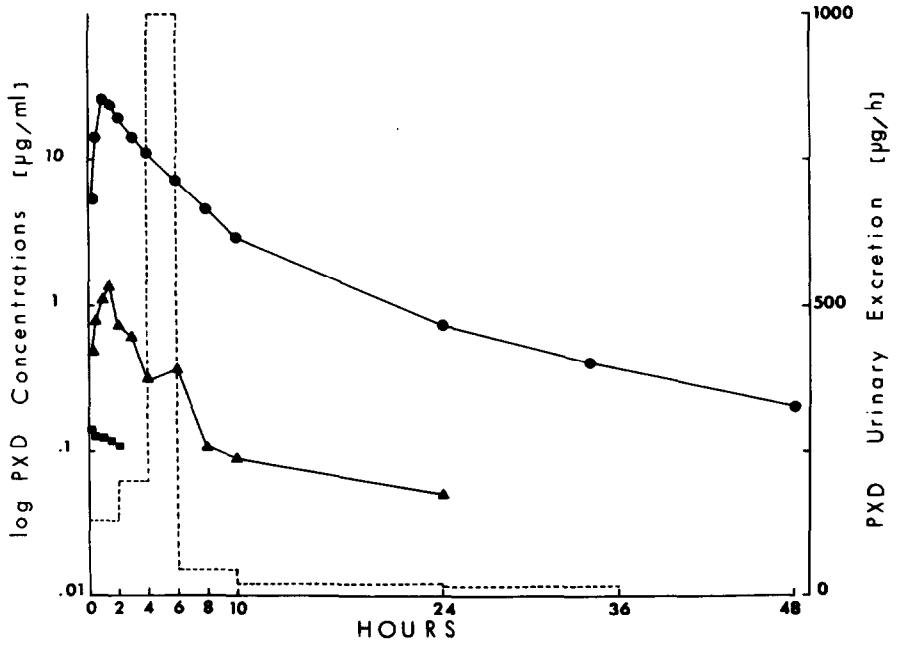

Fig. 2. Time course evolution of plasma (●), red blood cells (▲) and saliva (■) concentrations and urinary excretion (- - - -) of PXD in a healthy volunteer given 300 mg of PXD orally at 8.00 a.m.

example Fig. 2 shows the concentrations of unchanged PXD observed in plasma, red blood cells, saliva and urine from one healthy volunteer given 300 mg of PXD orally at 8.00 a.m. The time course of the evolution of PXD concentrations in plasma and red blood cells showed a biphasic decline and could be fitted as the sum of three exponentials. The saliva levels remained near the sensitivity limit of the method.

In conclusion, the method described is easy to use, selective, highly sensitive and reliable. Therefore, it appears to be suitable for following the concentrations of PXD after a single oral therapeutic dose in humans, and should be of value in determining the pharmacokinetic parameters of PXD and their possible alterations in pathological states such as renal insufficiency.

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