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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF CADRALAZINE FROM ITS POTENTIAL METABOLITES AND DEGRADATION PRODUCTS. QUANTITATION OF THE DRUG IN HUMAN PLASMA AND URINE

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SUMMARY

The chromatographic behaviour of cadralazine and its potential metabolites and degradation products with respect to pH, buffer molarity and composition of eluent is described. A selective method with an adequate sensitivity for the determination of the drug in human plasma and urine is also reported. The method includes extraction of biological fluids with chloroform and the analysis of extracts on a reversed-phase column with isocratic elution and detection at 254 nm. The method has been applied to the analysis of plasma and urine of a patient administered a single oral dose of 30 mg of cadralazine.

INTRODUCTION

Cadralazine (Fig. 1), ethyl 2-{6-[ethyl-(2-hydroxypropyl)amino]-3-pyridazinyl}hydrazinecarboxylate, is a new antihypertensive agent [1] which has shown its long-lasting activity in animals and man at very low dosages [2—6]. Studies of degradation in aqueous solutions [7] at different conditions of pH, temperature, concentration and in the presence or absence of light and oxygen, showed the formation of the following main degradation products: the pyridazine (PY), the pyridazinone (PYO) and the triazolone (TZO) derivatives (Fig. 1).

Metabolic investigations in rat and dog have shown that the drug is transformed into several metabolites [8, 9]. The main metabolites besides PY, PYO and TZO, are methyltriazole (MTZ), triazole (TZ), the N-deethyl derivative (PPY), the N-deisopropanol derivative (EPY), the oxopropyl derivative (OPY) and the deethoxycarbonyl derivative (HPY) (Fig. 1).

This paper describes a high-performance liquid chromatographic (HPLC)

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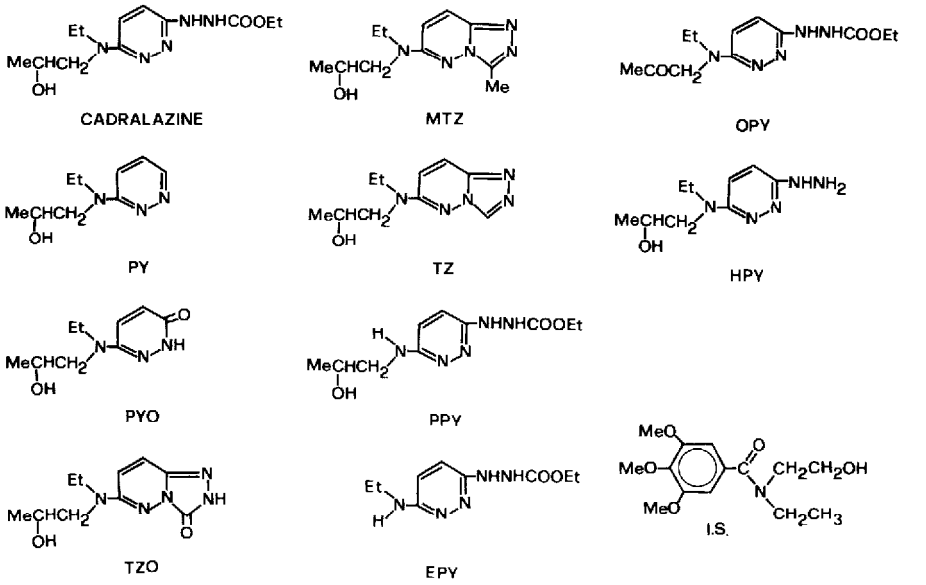

Fig. 1. Chemical structures of cadralazine, its degradation products, metabolites, and internal standard (I.S.).

separation of the parent drug from its main degradation products and metabolites, and its quantitation in human plasma and urine. An example from a pharmacokinetic study is also shown.

EXPERIMENTAL

Chemicals and glassware

Cadralazine, PY, PYO, TZO, MTZ, TZ, PPY, EPY, OPY, HPY and the internal standard (I.S., Fig. 1) were synthesized in our laboratories. Chloroform, methanol and acetonitrile (LiChrosolv) were obtained from Merck (Darmstadt, F.R.G.); double-distilled water, 0.05 *M* sulphuric acid, sodium dihydrogen phosphate, phosphate buffer pH 7.4, 0.1 *M* hydrochloric acid, and sodium bicarbonate were from Carlo Erba (Milan, Italy). Separating phase filters (Whatman, Maidstone, U.K.) were used after extraction with chloroform.

The test tubes were cleaned with sulphochromic mixture and then silanized with a 10% toluene solution of Surfasil (Pierce, Rockford, IL, U.S.A.).

Cadralazine and internal standard were dissolved in pH 7.4 buffer for the calibration curves as reported under assay procedure and under calibration curves.

Chromatographic conditions

The high-performance liquid chromatograph consisted of a Model 6000A pump, a Model Lambda Max 480 ultraviolet (UV) absorbance detector operating at 254 nm and a U6K injector, all from Waters Assoc. (Milford, MA, U.S.A.). The signal of the detector was recorded with an Omniscribe recorder from Houston Instruments (Austin, TX, U.S.A.) and integrated with an M2 integrator from Perkin-Elmer (Norwalk, CT, U.S.A.). A stainless-steel column,

Hibar RP-8, 10 μm (25 cm × 4 mm I.D., Merck) connected with a precolumn dry packed with Perisorb RP-8 30—40 μm (Merck) was used. The mobile phase consisted of acetonitrile—0.1 *M* sodium dihydrogen phosphate in double-distilled water brought to pH 6.0 with 0.1 *M* sodium hydroxide (15:85); the flow-rate was 2.5 ml/min for urine and 2.7 ml/min for plasma analysis.

Assay procedure in plasma

To 2-ml aliquots of human plasma, 1 ml of internal standard solution (621 ng/ml) was added and the mixture was brought to 5 ml with pH 7.4 phosphate buffer. Extraction was performed with 6 ml of chloroform by mechanical shaking for 15 min. After centrifugation at 2900 *g* for 5 min, the organic phase was filtered through a phase separator filter. The aqueous phase was extracted again with 6 ml of chloroform with the same procedure. The organic layers were collected and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 2 ml of methanol, vortexed, and then dried under nitrogen. Methanol (100 μl) and 0.05 *M* sulphuric acid (50 μl) were added and 50 μl were injected.

Assay procedure in urine

Aliquots of 1 ml of human urine were brought to 4 ml with water, added with 0.5 ml of 0.1 *M* hydrochloric acid and then extracted with 10 ml of chloroform by mechanical shaking for 20 min. After centrifugation at 2900 *g* for 3 min, the organic layer was discarded, the aqueous phase was added with 1 ml of a saturated solution of sodium bicarbonate and 1 ml of internal standard (12.43 μg/ml) and then extracted with 10 ml of chloroform as previously described. After centrifugation the organic layer was filtered through a phase separator filter, evaporated to dryness in the usual manner and reconstituted with 50 μl of 0.05 *M* sulphuric acid and 50 μl of methanol. Then 25 μl of the mixture were injected.

Calibration curves

Drug solutions in pH 7.4 phosphate buffer ranging from 800 to 60 ng/ml were used for plasma (S-1) and from 35 to 1 μg/ml for urine (S-2). Aliquots of 1 ml of S-1 were added to 2 ml of human plasma to obtain amounts of cadralazine in the range 400 to 30 ng/ml of plasma and aliquots of 1 ml of S-2 were added to 1 ml of human urine to obtain amounts from 35 to 1 μg/ml of urine. Then the samples were treated according to the assay procedures.

Study in man

A male patient (with essential hypertension) was orally given a single dose of 30 mg of cadralazine as a tablet. Blood samples collected at 0, 0.5, 1, 3, 6, 9 and 12 h after administration were mixed with heparin and centrifuged. The separated plasma was immediately frozen and stored at −20° C until the analysis. Urine was collected at 0, 0—3, 3—6, 6—9, 9—12, 12—24 and 24—30 h after administration; the volumes were recorded and aliquots of 50 ml of each sample were immediately frozen and stored at −20° C until analysis.

RESULTS AND DISCUSSION

Determination of chromatographic conditions

The goal of this study was to achieve separation between cadralazine and its potential metabolites and degradation products in order to selectively quantify the drug in biological fluids. Thus we have used C_8 reversed-phase hydrophobic chromatography and systematically studied different factors which control the retention of the examined compounds.

Effect of pH

Fig. 2 shows the pH dependence of the capacity factor k' for the examined compounds calculated in the usual way as the mean of at least three determinations. The range of pH values explored was between 2 and 7. It can be seen that for cadralazine, HPY, PY and MTZ the capacity ratios are sensitive to pH and this dependence is sigmoidal (with inflection points between pH 4 and 7).

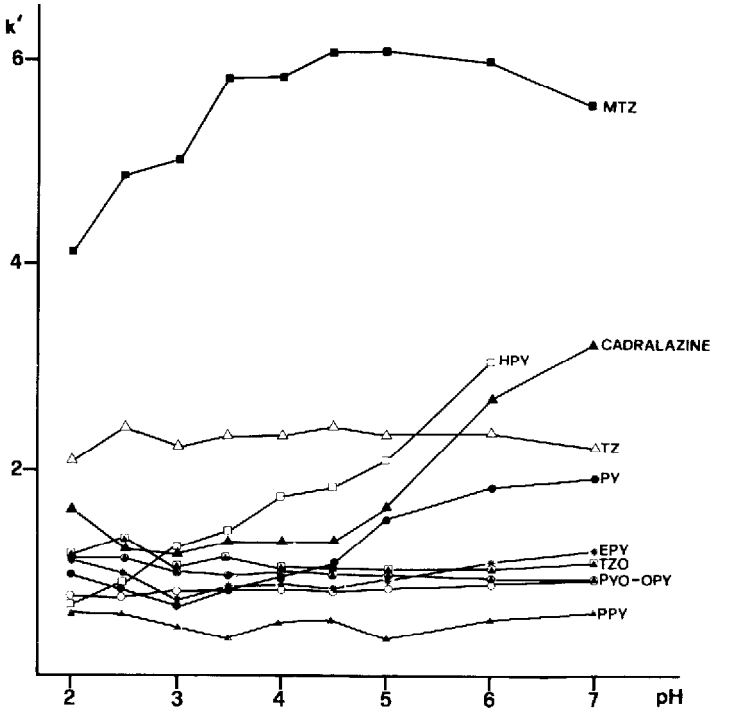

Fig. 2. Effect of pH on capacity ratio (0.1 *M* buffer—acetonitrile, 85:15).

This behaviour reflects the fact that, in general, the retention of weak bases from aqueous solutions, as a function of pH, follows their dissociation curves [10]. Consequently, the inflection points are located at $pH_m = pK_{am}$, i.e. when the pH of the mobile phase and the pK_a of the solute are equal. In Table I the pK_a values of all the compounds are reported. The best separations of the drug from its potential metabolites and degradation products were obtained at pH values higher than 5. Thus pH 6 was selected as the best compromise between selectivity and column stability.

TABLE I

pK_a VALUES OF CADRALAZINE AND ITS POTENTIAL METABOLITES AND DEGRADATION PRODUCTS IN AQUEOUS SOLUTION

Compound	pK_a*
Cadralazine	6.0
PYO	0.6
TZ	2.5
HPY	3.0 and 7.1
MTZ	4.5
PY	5.5
EPY	6.7
PPY	6.7
OPY	9.0
TZO	9.25

*Values were calculated by photometric titration.

Buffer molarity

The screening of the buffer molarity (Fig. 3) showed that the retention of all the considered products is largely influenced when the molarity is below 0.05, while it is practically insensitive above that value. Nevertheless at higher molarity the peaks sharpen, so the value of 0.1 *M* was selected in order to have the greatest sensitivity.

Effect of eluent composition

The composition of the eluent was explored between 10% and 20% of acetonitrile (Fig. 4). As expected, an increase in acetonitrile decreases the

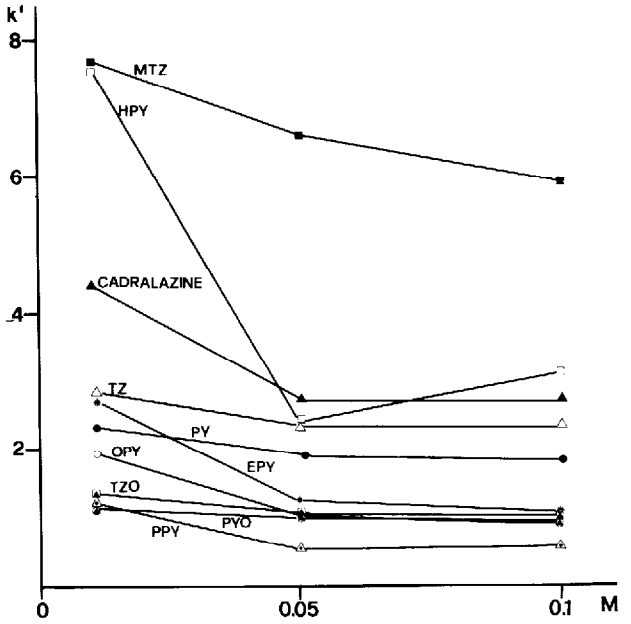

Fig. 3. Effect of buffer molarity on capacity ratio (buffer pH 6.0—acetonitrile, 85:15).

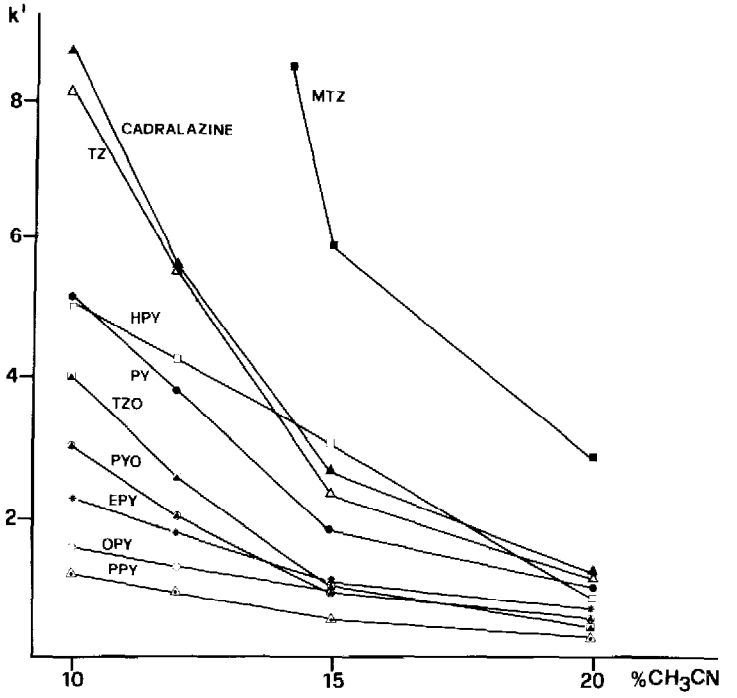

Fig. 4. Effect of eluent composition on capacity ratio (phosphate buffer 0.1 *M*, pH 6.0).

capacity ratios of all the compounds. Acetonitrile of 15% gives the best selectivity, particularly between cadralazine and TZ.

Chromatographic selectivity

On the basis of the foregoing, the chromatographic conditions reported in the experimental section were chosen. Under these conditions cadralazine elutes as a sharp peak and it is well separated from the other products (Fig. 5). No interference from naturally occurring endogenous compounds (either in plasma or urine) was observed. The chromatograms of plasma and urine blanks spiked with cadralazine and internal standard and of plasma and urine samples obtained from the patient after the oral administration of 30 mg of cadralazine are illustrated in Figs. 6 and 7, respectively.

Recovery

The recovery at different concentrations (extraction yield) was determined from the difference between the peak area ratio when cadralazine was added to plasma and urine (the internal standard being added to the final extracts) and the peak area ratio when both were added to the final extracts of blank plasma and urine. For the plasma the recovery was 87%, while for urine it was 65%; although the latter value was relatively low, due principally to the preextraction, it was nevertheless accepted because of its reproducibility over the whole calibration range. However, for the pharmacokinetic studies, single calibration curves were constructed for each subject using the individual urine blanks.

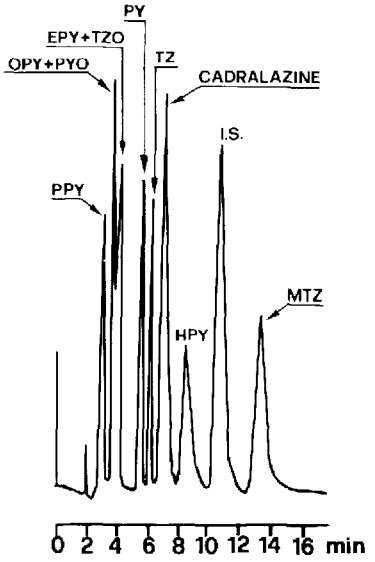

Fig. 5. HPLC separation of cadralazine from its degradation products, metabolites and internal standard (I.S.).

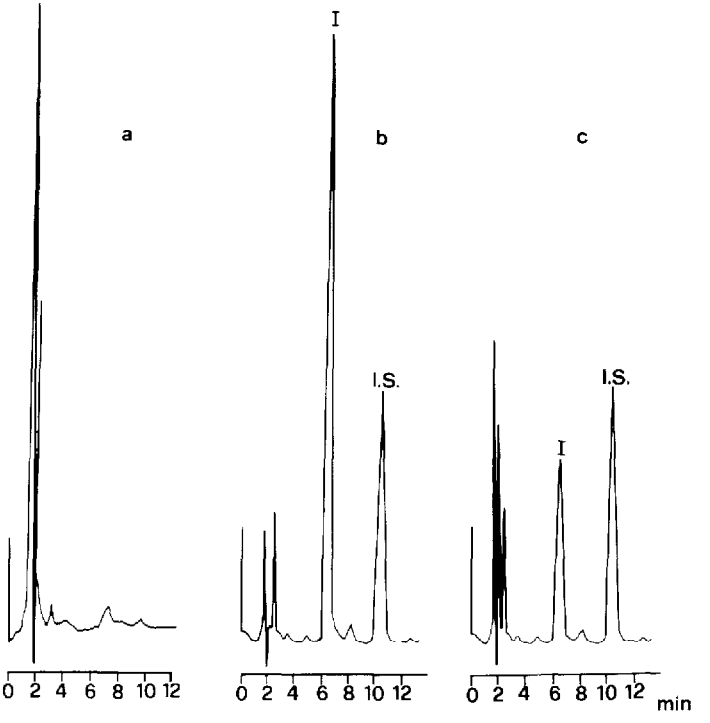

Fig. 6. HPLC profiles of (a) plasma blank, (b) plasma blank spiked with cadralazine (263 ng/ml) and internal standard (I.S., 310.5 ng/ml), and (c) a plasma sample 12 h after the administration of 30 mg of cadralazine to the patient.

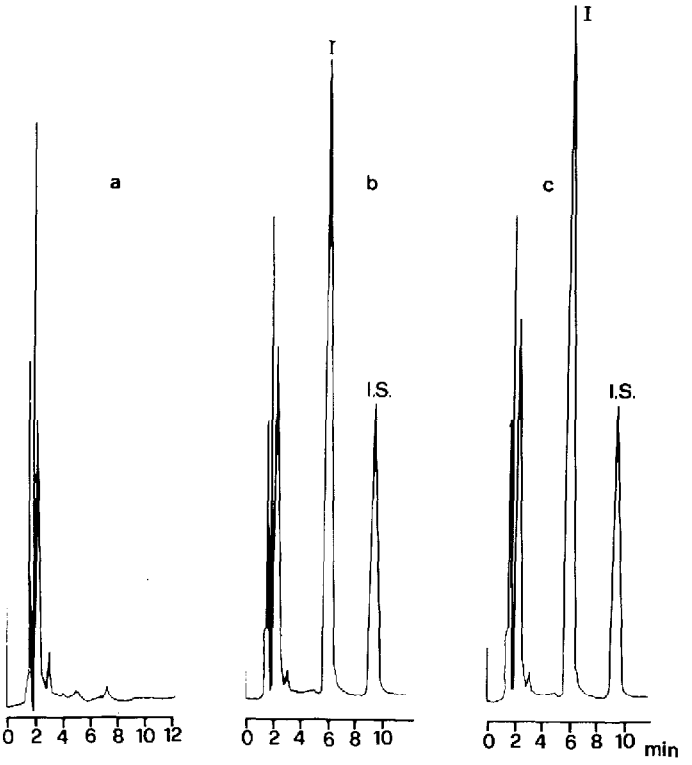

Fig. 7. HPLC profiles of (a) urine blank, (b) a urine blank spiked with cadralazine (7.02 μg/ml) and internal standard (I.S., 12.43 μg/ml), and (c) a sample of the patient's urine after administration of 30 mg of cadralazine.

TABLE II

VARIANCE ANALYSIS OF CADRALAZINE CALIBRATION CURVES IN BOTH PLASMA AND URINE

Data processed as described in ref. 11.

Source	Deviance	Degree of freedom	Variance	F calculated	F ($P < 0.05$) tabulated
Plasma					
Regression	16.07652	1	16.07652	2860.59	6.94
Residual	0.05616	10	0.00562		
Total	16.13268	11			
Lack of fit	0.02076	2	0.01038	2.35	6.06
Error	0.03540	8	0.00442		
Urine					
Regression	5.09171	1	5.09171	765.67	6.94
Residual	0.06652	10	0.00665		
Total	5.15823	11			
Lack of fit	0.01011	2	0.00506	0.72	6.06
Error	0.05641	8	0.00705		

Linearity of response and detection limits

The calibration curves (in the ranges mentioned above) were calculated using the peak area ratios versus drug concentrations by the method of least squares. The straight lines were: $Y = 0.07731 + 0.00720X$ for plasma; and $Y = -0.02410 + 0.18762X$ for urine. The goodness of fit of linear models was verified by analysis of variance [11] (Table II).

The detection limit for the drug in spiked plasma samples, based on a reproducibility of about 20%, was 10 ng/ml.

Reproducibility

The reproducibility of the method was evaluated over a concentration range of 50—330 ng/ml of plasma and over a concentration range of 1—35 μg/ml of urine. Each plasma specimen was analysed in triplicate over nine days within a period of about twelve weeks, while each urine specimen was analysed six times within a period of one week.

TABLE III

PRECISION AND ACCURACY IN THE DETERMINATION OF CADRALAZINE IN PLASMA AND URINE

Conc. added (plasma ng/ml; urine μg/ml)	Conc. found (plasma ng/ml; urine μg/ml)	C.V. (%)	Difference between found and added conc. (%)
Plasma (n = 3)			
99.7	97.3	3.45	−2.41
303.2	285.6	2.92	−5.80
404.4	391.9	0.84	−3.09
49.7	48.4	8.66	−2.62
187.0	187.0	0.75	0
331.0	338.2	2.31	+2.18
196.0	198.7	2.77	+1.38
216.0	217.7	1.29	+0.79
263.0	257.2	1.52	−2.21
Urine (n = 6)			
71.0	70.4	3.46	−0.8
28.2	27.9	2.42	−1.1
17.7	17.1	2.16	−3.4
1.4	1.3	4.21	−7.1

The data for precision (C.V. of replicate analyses) and accuracy (difference between found and expected concentrations) are presented in Table III.

Application

The described method was used for the determination of cadralazine in both plasma and urine of a subject administered the drug. The cumulative urinary excretion and the plasma levels found are shown in Fig. 8.

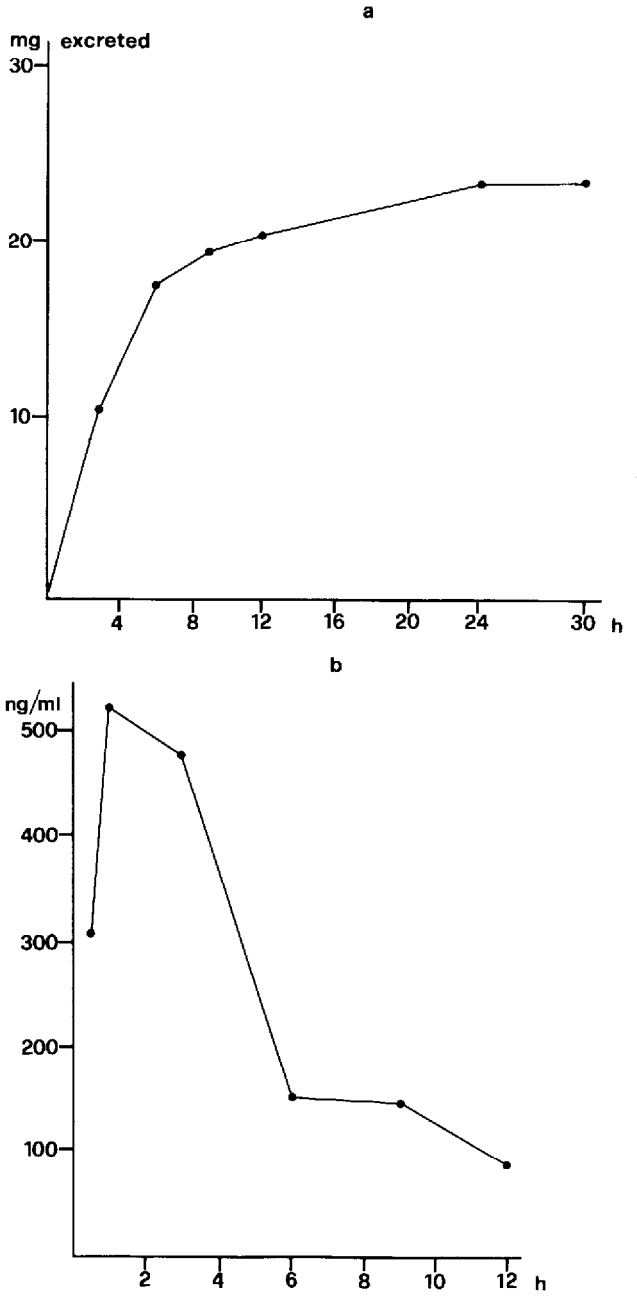

Fig. 8. Urinary cumulative excretion (a) and plasma levels (b) of cadralazine (30 mg, single oral dose).

CONCLUSIONS

A simple, sensitive, precise and accurate HPLC technique for the determination of cadralazine in human plasma and urine has been developed and it can be applied to routine analysis. The drug is well separated from its

metabolites and potential degradation products. The lower limit of sensitivity is adequate to follow the absorption and distribution of cadralazine in plasma and urine of human subjects administered a therapeutic dose of the drug, which lies between 15 and 30 mg, once a day.

REFERENCES

1 F. Parravicini, G. Scarpitta, L. Dorigotti and G. Pifferi, Farmaco. Ed. Sci., 34 (1979) 299.
2 C. Semeraro, L. Dorigotti, S. Banfi and C. Carpi, J. Cardiovasc. Pharmacol., 3 (1981) 455.
3 P. Van Brummelen, F.R. Bühler, W. Kioroski, P. Bolli and O. Bertel, Int. J. Clin. Pharmacol. Biopharm., 17 (1979) 380.
4 F.W. Amann and F.R. Bühler, Clin. Eur., 18 (1979) 1028.
5 G.C. Lavezzaro, D. Gastaldo, P. Noussan, M. Bensoni, J. Parini and P.F. Angelino, Minerva Cardioangiol., 29 (1981) 527.
6 P.F. Angelino, M. Bensoni, D. Gastaldo, M. Minelli, J. Parini and G.C. Lavezzaro, Minerva Cardioangiol., 30 (1982) 69.
7 M. Visconti, L. Citerio, M. Borsa and G. Pifferi, J. Pharm. Sci., (1984) in press.
8 L. Simonotti, M. Zanol, F. Parravicini and G. Pifferi, Eur. J. Drug Metab. Pharm., 7 (1982) 39.
9 M. Visconti, C. Farina, M. Pinza and G. Pifferi, Abstracts, IXth European Workshop on Drug Metabolism, Pont-à-Mousson, June 11—15, 1984, p. 35.
10 C. Horváth, W. Melander and I. Molnár, Anal. Chem., 49 (1977) 142.
11 J.R. Green and F. Margerison, Statistical Treatment of Experimental Data, Elsevier, Amsterdam, 1978, pp. 281—298.