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Note

Determination of metformin and phenformin in human plasma and urine by reversed-phase high-performance liquid chromatography

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The biguanide derivatives, particularly phenformin (N-phenethylbiguanide) and metformin (N,N-dimethylbiguanide), are currently used in the treatment of non-insulin-dependent diabetes mellitus [1] and hypertriglyceridaemia [2]. To date, the qualitative and quantitative determination of such drugs in biological fluids is still troublesome Most of the described methods suffer from a lack of selectivity and relatively low sensitivity [3—7]; others require complicated preparations of the sample to be analysed [8, 9].

Recently, high-performance liquid chromatography (HPLC) has been used for the assay of metformin and phenformin in blood and urine [10—13]. However, the reported HPLC procedures still necessitate the derivatization of metformin [10] and are not conclusive as far as sensitivity, precision, specificity and/or application to human subjects are concerned [11, 12] Finally, it is not known if the cation-exchange HPLC method described in ref 13 can be used for phenformin as well as for metformin

In this paper we describe a rapid, reproducible and precise method to quantitate both phenformin and metformin in human plasma and urine by reversed-phase HPLC, moreover, the selectivity and sensitivity of the procedure is demonstrated Finally, the results obtained from analysis of plasma and urine samples from a group of diabetic patients are reported.

EXPERIMENTAL

Materials

Chemicals and solvents were obtained from Merck (Darmstadt, F.R.G.)

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and were of HPLC grade. Deionized and distilled water, successively purified by the Milli-Q system (Millipore, Bedford, MA, U S A), was used for reagent preparations. Solvent mixtures were degassed by vacuum and filtered through a 0 22-μm pore size filter (Millipore) before use Metformin and phenformin were kindly provided by Laboratori Guidotti (Pisa, Italy)

HPLC

A Waters Model 510 pump (Waters Assoc., Milford, MA, U.S A) and a Waters absorbance detector Model 441 UV monitor were used Injections of 200 μl were made with a Waters U6K liquid chromatography injector filled with a 2-ml loop.

Isocratic separations were performed at room temperature on a prepacked 300 × 3.9 mm I D. C_{18} μBondapak (10 μm) reversed-phase liquid chromatography column obtained from Waters and monitored by UV detection at 254 nm, moduling the amplification from 0 005 to 0.2 The mobile phase consisted of 0 01 *M* phosphate buffer—acetonitrile (50·50)

Standard and sample preparation

The stock solutions of metformin and phenformin were prepared by dissolving the drugs in distilled water to obtain a final concentration of 1 mg/ml. By successive dilutions of the stock solution, the standard solutions were obtained (concentrations ranging from 25 to 5000 ng/ml for phenformin and from 75 to 10 000 ng/ml for metformin).

Plasma and urine of healthy subjects and diabetic patients treated with metformin or phenformin were also analysed. In 0 3-ml aliquots of plasma from healthy subjects, 10 μl of the stock solution of metformin or phenformin were added before or after deproteinization with acetonitrile (final ratio 1 5), centrifugation was successively performed at 2000 *g* for 15 min [14]. After centrifugation, the solvent was evaporated from the supernatant Blood of diabetic patients was drawn basally and 2 h after the last ingestion of 500 mg of metformin or 30 mg of phenformin, and plasma was deproteinized as described above Urine samples (0 3 ml, previously adjusted to pH 8 with 1 *M* potassium hydroxide) were treated in a similar manner to plasma In diabetic patients, 24-h urine specimens were analysed.

RESULTS

Reversed-phase HPLC of plasma from healthy subjects to which biguanide had been added before the deproteinization showed the presence of a peak with a retention time of 8 min (k' = 3 1) for metformin (Fig 1B) and 14 9 min (k' = 6 5) for phenformin (Fig 2B)

The same retention times were obtained when plasma deproteinization was performed before the addition of biguanides, and the areas under the peaks differed less than 5% from those obtained after the former procedure Thus, plasma deproteinization in the presence of the drugs is not accompanied by a significant loss of biguanides from the supernatant

The retention times of metformin and phenformin obtained by HPLC of plasma from the diabetic patients were the same as those observed when the

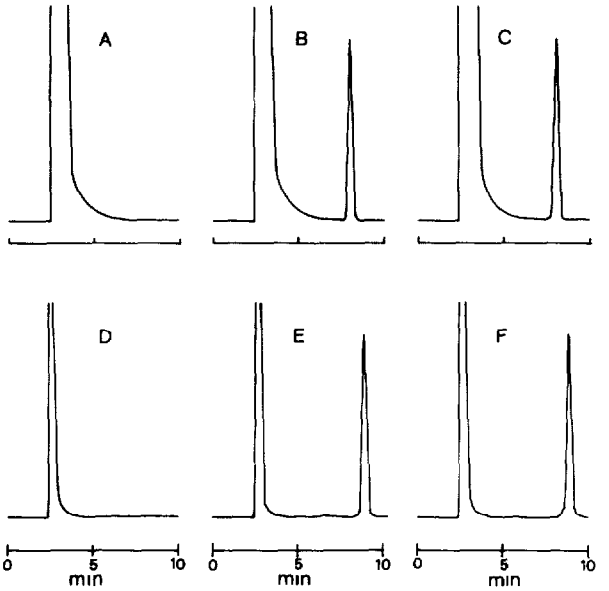

Fig 1 Chromatograms of blank plasma (A), blank plasma supplemented with 33 μg/ml metformin (B), patient's plasma containing metformin (C), blank urine (D), blank urine supplemented with 33 μg/ml metformin (E) and patient's urine (F)

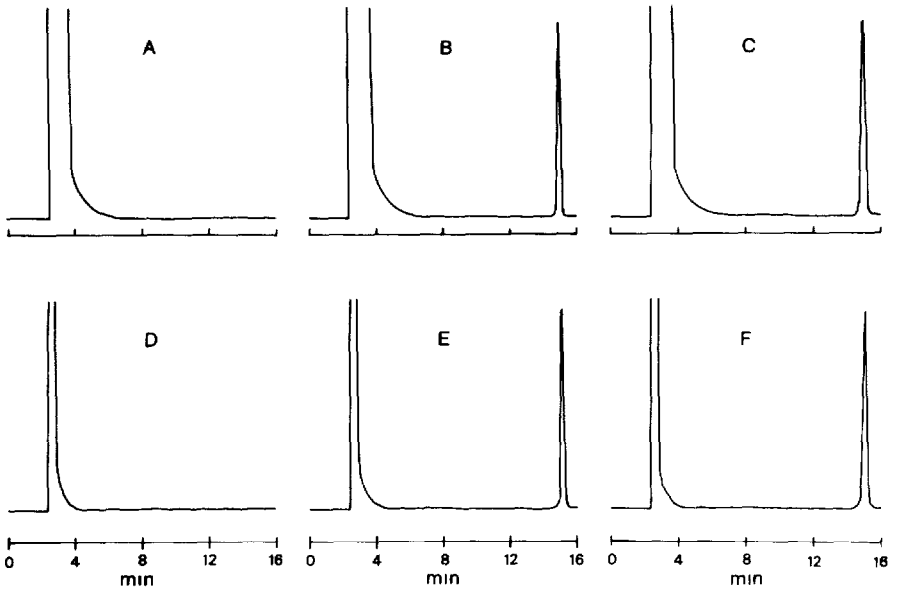

Fig 2 Chromatograms of blank plasma (A), blank plasma supplemented with 33 μg/ml phenformin (B), patient's plasma containing phenformin (C), blank urine (D), blank urine supplemented with 33 μg/ml phenformin (E) and patient's urine (F)

plasma from healthy subjects to which the biguanides had been added was used as the standard (Fig 1C, Fig 2C).

HPLC analysis of urine from healthy subjects and diabetics is also shown in Figs 1 and 2 (D, E and F in each). The retention times were 8.80 min (k' =

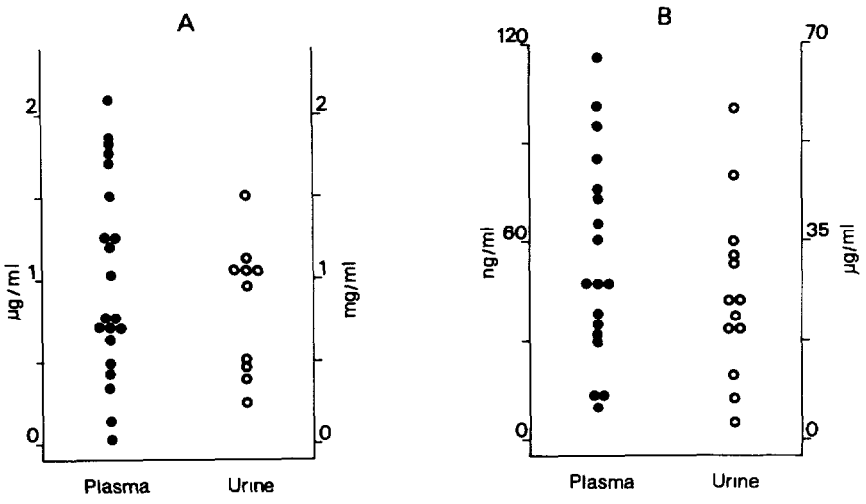

Fig 3 Plasma (•) and urine (○) concentrations of metformin (A) and phenformin (B) in diabetic patients

TABLE I

DRUGS BEING RECEIVED BY PATIENTS WHOSE PLASMA AND URINE WERE ANALYSED FOR METFORMIN (A) AND PHENFORMIN (B)

A	B
Amiloride	Amiloride
Bromexine	Bromazepam
Chloropropamide	Chlorpropamide
Clonidine	Digoxin
Diazepam	Dipyridamole
Digoxin	Erythrityl tetranitrate
Dipyridamole	Ethacrinic acid
Ethambutol	Ethambutol
Flunarizinum	Flunarizinum
Flunitrazepam	Flunitrazepam
Furosemide	Gangliosidum complex
Gangliosidum complex	Glyburide
Glyburide	Insulin
Hydrochlorothiazide	Nifedipine
Indapamidum	Nortriptyline
Insulin	Pentossiphylline
Isosorbide dinitrate	Pipemidic acid
Mianserinum	Piperazine
Nifedipine	Spironolactone
Nortriptyline	Sulfamethoxazole
Piperazine	Trimethoprim
Ranitidinum	Verapamil
Rociverinum	Vitamin B complex
Spironolactone	
Verapamil	
Vitamin B complex	
Triazolam	

3.4) and 15.10 min (k' = 6.6) for metformin and phenformin, respectively. The coefficients of variation of the capacity factors for metformin and phenformin were less than 3% for both within- and between-assay.

The linearity of the method for metformin and phenformin was tested by plotting the peak heights of metformin and phenformin chromatographed by HPLC against their concentrations in standard solutions Both for metformin (range 75—10 000 ng/ml) and phenformin (range 25—5000 ng/ml), the plots were linear (r = 0.99) and passed through the origin The within-assay coefficient of variation of the peak heights was 4.5% for metformin and 3% for phenformin, at low and high concentrations, the between-assay coefficient of variation for both drugs was computed by testing the samples during three days of storage at 4°C, resulting in 10 and 4.3% for metformin and phenformin, respectively

The analytical procedure was successively applied to the plasma and 24-h urine samples from a group of non-insulin-dependent diabetic patients. As shown in Fig. 3, the assay measured the concentration of phenformin and metformin when the drugs are administered at the usual therapeutic dosage, thus demonstrating its usefulness in clinical performance. The limits of detection of metformin and phenformin were 50 and 12 5 ng/ml, respectively

In order to test the selectivity of the analysis, we chromatographed plasma samples from patients taking the drugs listed in Table I, and no interference was observed between such drugs and phenformin and metformin.

DISCUSSION AND CONCLUSION

Metformin and phenformin are used clinically as oral hypoglycaemic agents in non-insulin-dependent diabetes mellitus [1]; moreover, a hypolipidaemic effect of metformin in hypertriglyceridaemic patients has been shown [2] However, both drugs, especially phenformin, alter the lactate metabolism, and a number of cases of lactic acidosis in patients assuming the two biguanides have been reported [15] As the development of lactic acidosis is often correlated with drug plasma levels [16], a rapid, selective and sensitive method to determine the concentration of metformin and phenformin in body fluids is highly advisable

In this paper we have reported a procedure for the assay of the two drugs in human plasma and urine by reversed-phase HPLC The procedure allows measurement of the plasma and urine levels of metformin and phenformin in a rapid, sensitive and selective way, appearing to be a good option for the monitoring of patients on chronic biguanide therapy

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