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DETERMINATION OF ORAL ANTI-DIABETIC AGENTS IN HUMAN BODY FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Two widely prescribed anti-diabetic agents for which no simple assay method was previously available can now be determined by high-performance liquid chromatography using a UV detection system. The two drugs investigated were tolbutamide (a sulphonylurea) and phenformin (a biguanide).

Tolbutamide can be assayed directly, after a single extraction step, on a reversed-phase system, illustrating the simplicity of the technique for carrying out analyses on underivatised drug compared with gas chromatography.

Phenformin was not so easily chromatographed using straightforward partition systems; however, by the choice of a suitable ion-pair agent it was possible to chromatograph the underivatised drug in a relatively simple reversed-phase system.

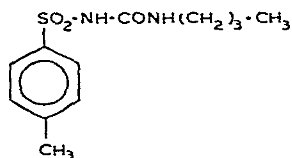
INTRODUCTION

There are two major groups of orally active hypoglycaemic agents, namely the sulphonamide derivatives and the biguanides. Of these, tolbutamide (Rastinon; Hoechst) is representative of the former group and phenformin (Dipar; Hoechst) is an example of the latter. In order to elucidate further their mechanisms of action and pharmacokinetics in human volunteers, it is necessary to monitor their drug plasma concentrations.

A review of the existing methods available suggested that they were not sensitive enough after therapeutic doses were given or else the equipment was sophisticated, permitting only a small daily throughput of samples. In order to analyse the rationale behind the assays developed, the drugs are discussed separately.

EXPERIMENTAL AND RESULTS

Tolbutamide (N-butyl-N′-toluene-p-sulphonylurea)



Both colorimetric and gas chromatographic (GC) methods exist for the determination of sulphonylureas. The colorimetric methods are based on hydrolysis of the drug, extraction of the liberated amine and analysis of the amine using dinitrofluorobenzene, ninhydrin[1,2] or the Bratton and Marshall reaction[3]. These methods are neither specific nor very sensitive.

GC of the intact sulphonylurea has been unsuccessful mainly because of thermal lability. Methylation of tolbutamide to facilitate GC has been carried out using dimethyl sulphate[4] or diazomethane[5]. However, the derivatives so formed cleave on injection into the injection port to give N-methyl-*p*-toluenesulphonamide, N-methyltolbutamide and a methyl enol ether of tolbutamide[4]. By careful choice of the injection port temperature, however, breakdown of the N-methyl derivative can be minimized.

Flash heater methylation[6] using tetramethylammonium hydroxide produces one peak, the N,N-dimethyl derivative[7]. However, *p*-toluenesulphonamide, a metabolite of tolbutamide, also undergoes flash heater methylation to an N,N-dimethyl derivative, which interferes with the analysis.

Thus, the colorimetric determinations were rejected on the grounds of specificity and sensitivity. Although the GC methods presented no sensitivity problems, interference from metabolites was a possibility. This, coupled with the inherent instability of the derivatives, suggested the need for a rapid, sensitive, selective, alternative to GC. Such a requirement could be fulfilled by high-performance liquid chromatography (HPLC).

Extraction procedure. Plasma (1.0 ml) and 1 *N* hydrochloric acid (1.0 ml) were mixed together with dichloromethane (7 ml) on a Heto Rotamix for 10 min, centrifuged and the aqueous layer was aspirated. An aliquot (5 ml) of the dichloromethane layer was removed and evaporated to dryness under a stream of nitrogen at 40°.

The dried residue was dissolved in 100 or 200 μl of methanol and 20 μl of the solution were injected on to the column.

HPLC conditions. A Waters M6000 pump, a Waters U6K injector, a Waters μBondapak C_{18} column and a Cecil CE 212 variable-wavelength detector (228 nm) were used. The mobile phase was methanol–0.2% acetic acid (3:2) at a flow-rate of 1.2 $ml \cdot min^{-1}$. The chart speed was 0.5 $cm \cdot min^{-1}$.

Choice of detector wavelength. Tolbutamide in the mobile phase has an absorbance maximum at 228 nm with a sharp fall-off before and after the peak. Because of the sharp peak, it is essential to maintain an accurate wavelength setting in order to obtain reproducibility.

Choice of mobile phase. As tolbutamide is a weak acid, it is desirable when using a reversed-phase column tc suppress the ionization of the compound; an acidic phase was therefore chosen. The relative amounts of methanol and acetic acid are determined by varying the proportion of methanol to yield a retention time commensurate with a short assay time and good separation from endogenous plasma components. The extent of the interfering peaks is shown in Fig. 1.

Linearity. The method is linear up to 5 μg of tolbutamide injected. Consequently, extracts are taken up in a volume of dichloromethane to give a concentration of less than 5 μg of tolbutamide per 20 μl.

Calibration graphs produced after dissolving standard extracts in methanol produced a linear correlation of absorbance with concentration. Altering the volume

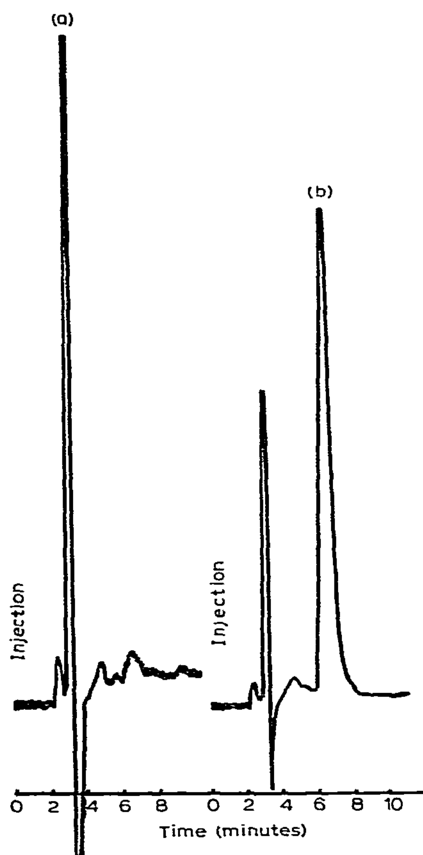


Fig. 1. Chromatogram of a plasma extract in the presence and absence of tolbutamide. (a) Blank plasma (0.02 a.u.f.s.), 0.1 ml of plasma extracted. (b) Plasma containing 27 $\mu g \cdot ml^{-1}$ of tolbutamide (0.05 a.u.f.s.), 0.1 ml of plasma extracted (equivalent to *ca.* 540 ng of tolbutamide injected).

of plasma used made a reduction in the slope of the calibration graph of 10% for 1 ml of plasma, compared with 0.1 ml plasma extracted. This may be accounted for by a decrease in the amount of lipid extracted, thereby facilitating the dissolution of the tolbutamide in methanol.

Choice of extraction conditions. As tolbutamide is a weak acid and dichloromethane is a relatively polar solvent, it is probable that it would be extracted over a broad pH range. Thus, in order to compare extractability and plasma backgrounds, known amounts of tolbutamide added to plasma were extracted under different conditions (1 *N* hydrochloric acid, pH 3.5 and pH 7.5). Extraction from plasma acidified with 1 *N* hydrochloric acid gave maximum extraction of tolbutamide without significant interference from endogenous plasma components, although less plasma background was observed at pH 7.5.

Stability. Samples, once extracted and stored dry at 0–4°, were stable for at least 3 days, as judged by comparison with freshly prepared standards using HPLC.

Sensitivity. The method is sensitive to 200 $ng \cdot ml^{-1}$ of tolbutamide in plasma.

Selectivity. No interfering peaks were observed in the plasma of about 20 human subjects examined. The possibility of other drugs interfering has not been investigated.

Precision. Precision was determined by using the equation of Snedecor[8]:

$$s = \sqrt{\frac{\Sigma d^2}{2n}}$$

where d is the difference between duplicates and n is the number of duplicates. For 30 duplicate determinations over the range 0.5–to 110 μg·ml^{-1}, $s = 1.8\%$.

Sample size. Using a Waters U6K injector, it was found that the injection of samples of 20 or 50 μl resulted in peak broadening with a consequential loss in peak height of about 10–20%. Consequently, all injections were made with a constant volume of 20 μl.

Application. The method has been applied to human volunteers taking part

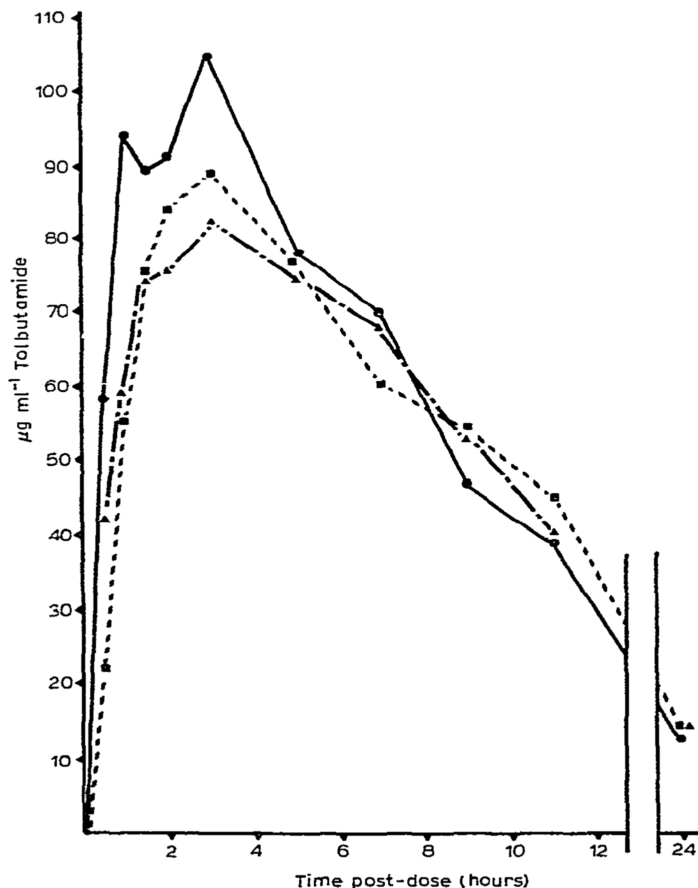
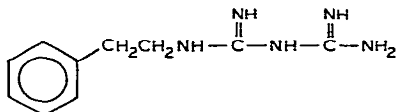


Fig. 2. Plasma profile of six volunteers dosed with three different preparations of tolbutamide (Rastinon, 1 g). Each point is the mean for the six volunteers.

in a bioavailability study. Three preparations were compared in a cross-over study using six volunteers. A summary of the plasma profiles obtained for the three preparations is shown in Fig. 2.

Phenformin (N-phenethylbiguanide)



Thin-layer chromatographic (TLC)[9] and paper electrophoresis[9,10] methods exist for the separation of phenformin. However, none of these is sensitive enough to measure phenformin in plasma after therapeutic doses, nor are the colorimetric methods of Garrett and Tsau[11] and Shepherd and McDonald[12]. However, a gas chromatographic–mass spectrometric (GC–MS) method[13] for measuring the *s*-triazene derivative has been described. This method is sensitive enough for measuring concentrations in human plasma. However, the equipment requirement is not generally available in a routine analytical laboratory. Again, HPLC presented a possible solution to this problem; the main aim in its application was to achieve as simple and sensitive a method as possible.

Extraction procedure for plasma. To plasma (1 ml) in a centrifuge tube are added 1 *N* acetic acid (0.1 ml) and methanol (2 ml). The tube is vortex mixed for 30 sec and centrifuged at 1000 *g* for 15 min. The supernatant containing the drug is removed and transferred into a 15-ml Sovirel tube, made alkaline by addition of 3 *N* sodium hydroxide solution (1 ml) and extracted with dichloromethane (12 ml) for 10 min on a tumble mixer. After centrifugation for 10 min at 1500 *g*, an aliquot of the dichloromethane layer (10 ml) is transferred into a tapered centrifuge tube, 1 *N* acetic acid (0.1 ml) is added and the dichloromethane is evaporated. The dried residue is dissolved in 50 μl of 0.1 *N* acetic acid–methanol (1:1) and 25 μl of the solution are chromatographed (Fig. 3).

Extraction procedure for urine. Urine (1 ml) in a Sovirel tube is made alkaline with 3 *N* sodium hydroxide solution (2 ml) and extracted with dichloromethane (12 ml) using a tumble mixer. The tube is centrifuged, an aliquot of the dichloromethane layer is transferred into another Sovirel tube, 0.1 *N* acetic acid (2 ml) is added and the tube is tumble mixed for 10 min. Following centrifugation, 25 μl of the aqueous layer are chromatographed (Fig. 4).

HPLC conditions. The instrumentation was the same as for tolbutamide, except that also a Pye LC 3 variable-wavelength detector (235 nm) was used. The mobile phase was methanol–water (1:1) containing 0.02% of acetic acid and $5 \cdot 10^{-3}$ *M* 1-heptanesulphonic acid, at a flow-rate of 1 $ml \cdot min^{-1}$. The chart speed was 0.5 $cm \cdot min^{-1}$.

Choice of detector wavelength. In the above mobile phase, phenformin has an absorbance maximum at 233 nm.

Choice of mobile phase. Phenformin is a strongly basic compound ($pK' \approx 3$ and $pK'' \approx 13$) and is partially ionised at pH 8 (the maximum operating pH for silica columns). Thus, it would not be expected to be chromatographed well on reversed-phase columns because of this pH constraint; the best chromatogram and conditions

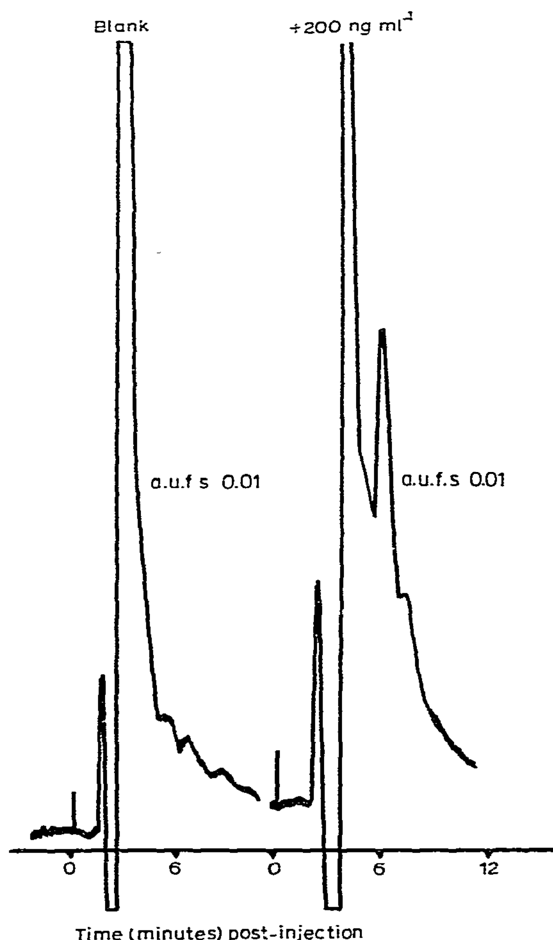


Fig. 3. Chromatogram of plasma extracts with 200 ng·ml^{-1} of and without phenformin.

for a reversed-phase system are shown in Fig. 5. However, by incorporating an ion-pair agent into the system, and decreasing the pH, good chromatography was achieved (Fig. 6). Although it was possible to chromatograph this compound on an ion-exchange column, the co-extracted material from plasma masked the phenformin peak.

The plasma residue was dissolved in 50 μl of 0.1 N acetic acid–methanol (1:1).

Linearity. The method is linear over the range covered for both plasma and urine (up to 200 ng injected).

Extraction conditions. Because phenformin is a water-soluble strong base, it should be possible to extract it from alkaline solution into dichloromethane. However, because the plasma proteins are denatured by dichloromethane and denatured proteins co-precipitate biguanides[14], some means of removing the proteins while leaving the phenformin in solution was sought. This was achieved by precipitation of acidified plasma with methanol, followed by alkalinization and extraction of the drug with dichloromethane[11].

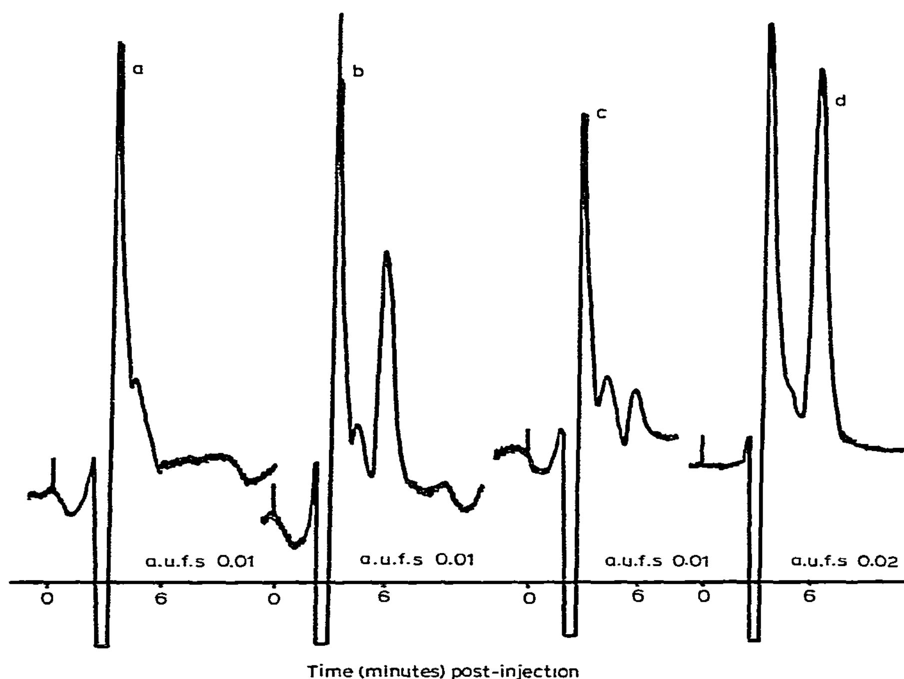


Fig. 4. High-performance liquid chromatograms of urine extracts. (a) Urine blank; (b) urine blank + 5 $\mu g \cdot ml^{-1}$ of phenformin; (c) 24–36-h urine sample; (d) 12–24-h urine sample.

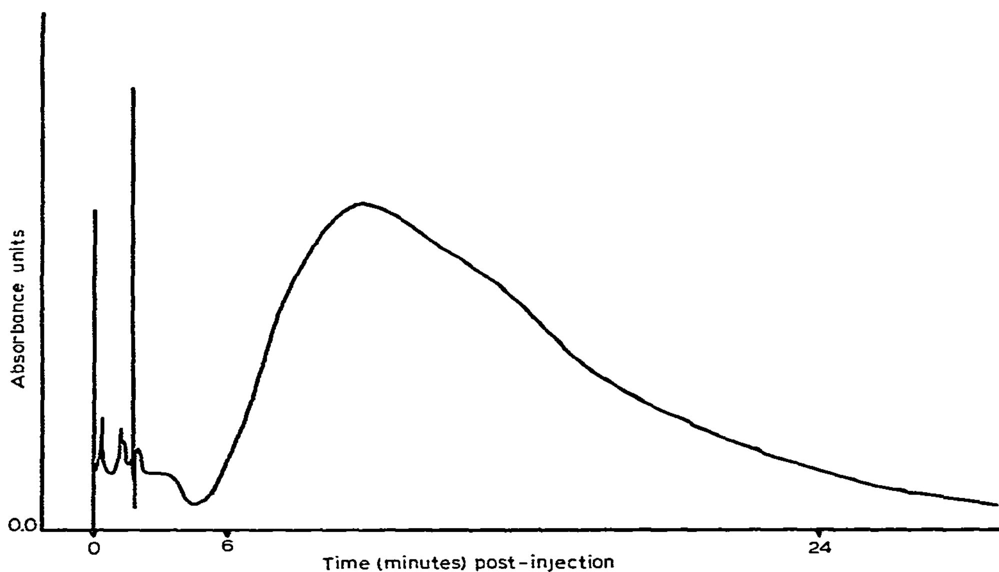


Fig. 5. Effect of absence of an ion-pair agent on the chromatographic properties of phenformin. Column: μBondapak C_{18}. Pump: Waters M6000, flow-rate 1.0 $ml \cdot min^{-1}$. Mobile phase: methanol–0.1% ammonium carbonate solution (4:1), pH 8. Detection: Cecil CE 212 UV (235 nm), a.u.f.s. 0.02. Sample: 2 μg of phenformin.

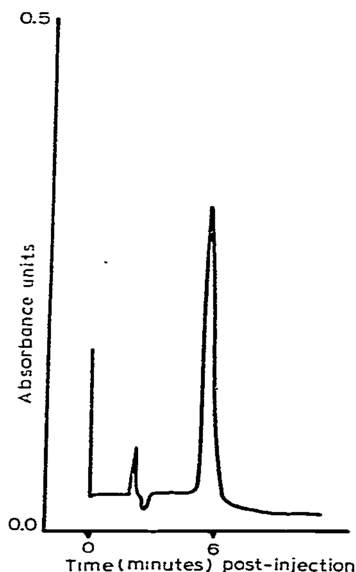


Fig. 6. Effect of addition of an ion-pair agent on the chromatographic properties of phenformin. Column: μBondapak C_{18}. Pump: Waters M6000, flow-rate 1.0 ml·min^{-1}. Mobile phase: methanol–water (1:1) containing 0.02% of acetic acid and $5 \cdot 10^{-3}$ *M* 1-heptanesulphonic acid (pH 3.7). Detection: Cecil CE 212 UV (235 nm), a.u.f.s. 0.05. Sample: 2 μg of phenformin.

Extraction from urine was simpler because of the high concentration of the drug and the absence of protein. Back-extraction into dilute acetic acid was found to reduce background interference.

Stability. Extracted plasma samples had to be analysed immediately, whereas urine extracts in acetic acid were stable for at least 2 days at 0–4°, as judged by HPLC.

Sensitivity. The method is sensitive to 250 ng·ml^{-1} of phenformin in urine, although this could be improved if required by using smaller volumes of acetic acid for the back-extraction. For plasma, the method is sensitive to 10 ng·ml^{-1} of phenformin.

Selectivity. Interference from endogenous plasma or urine components was not observed in either plasma or urine extracts of the three subjects investigated.

Precision. Because of the small number of samples analysed, no attempt has been made to produce an *s* value according to the equation of Snedecor.

Sample size. Samples injected were of the same volume (25 μl).

Application. The method has been applied to one human volunteer who was dosed with 100 mg of Dipar. The results (Fig. 7) are similar to those previously reported using GC–MS[15].

CONCLUSION

HPLC has provided a simple means of measuring the plasma levels of two important drugs which hitherto have been fraught with problems or required sophisticated instrumentation. Merely by adjusting the type and components of the mobile

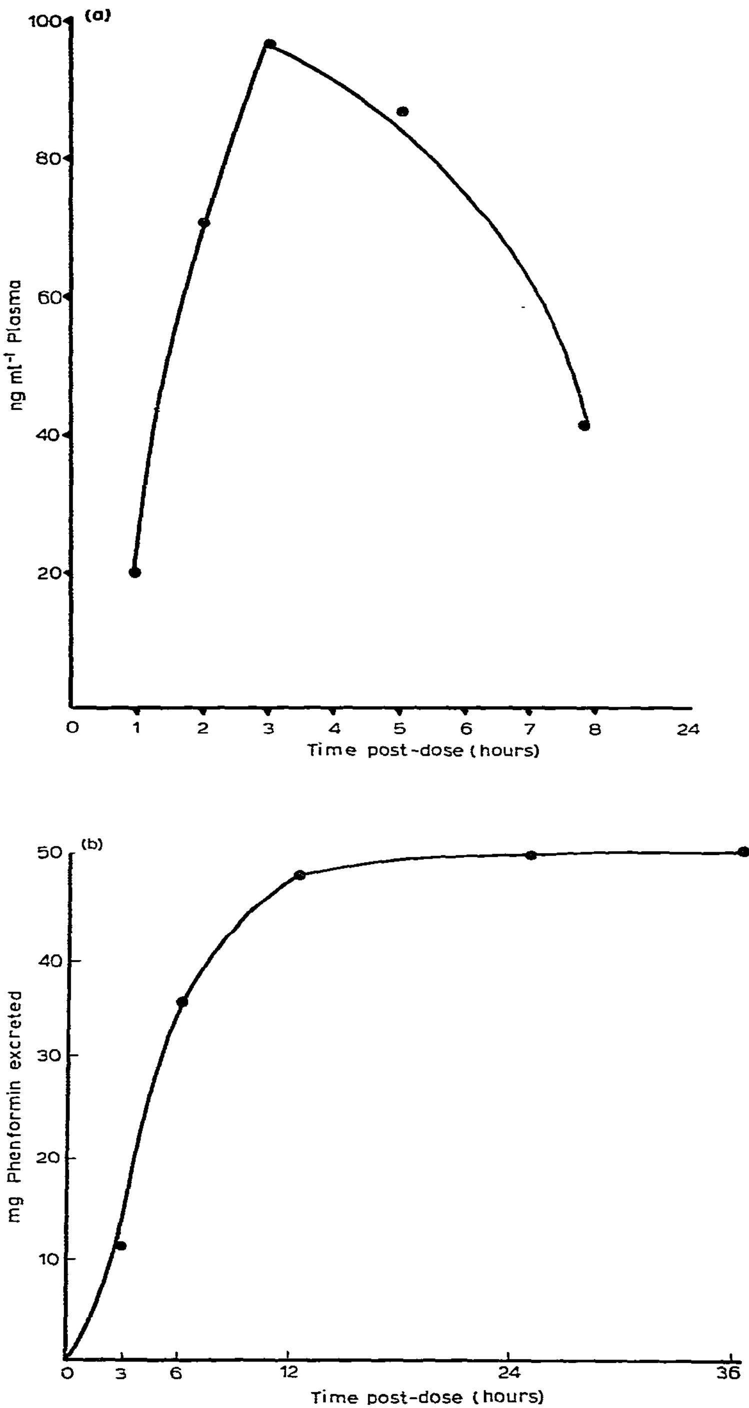


Fig. 7. (a) Plasma profile of a volunteer dosed with Dipar (100 mg of phenformin). (b) Cumulative excretion of phenformin in urine of a volunteer dosed with Dipar (100 mg of phenformin).

phase it has been possible to use the same ODS bonded silica column for two distinctly different chemical moieties. It is this universal application of HPLC and the use of ODS bonded columns that have increased the popularity of HPLC.

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