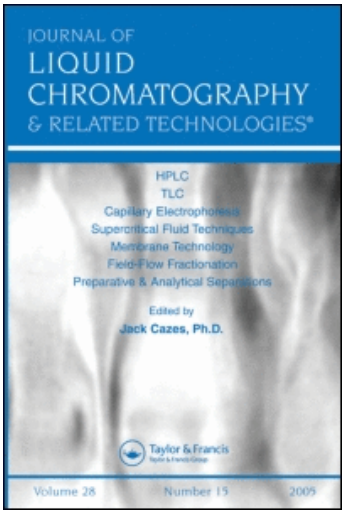

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C. Arcelloni[a]; I. Fermo[a]; R. Paroni[a]; A. Calderara[b]; M. Pacchioni[b]; A. E. Pontiroli[b]
[a] Istituto Scientifico Ospedale San Raffaele, Milano, Italy [b] Cattedra di Clinica Medica Universita'di Milano, Milano, Italy

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GLIBENCLAMIDE AND TOLBUTAMIDE IN HUMAN SERUM: RAPID MEASUREMENT OF THE FREE FRACTION

C. ARCELLONI[1], I. FERMO[1],
A. CALDERARA[2], M. PACCHIONI[2],
A. E. PONTIROLI[2], AND R. PARONI[1]
[1]*Istituto Scientifico Ospedale San Raffaele*
Via Olgettina 60
Milano, Italy
[2]*Cattedra di Clinica Medica*
Universita'di Milano
Milano, Italy

ABSTRACT

A rapid and reproducible procedure to evaluate the glibenclamide and tolbutamide free fraction in serum was developed. The ultrafiltration technique was used, employing disposable filter units (Centricon 30, Amicon) with 30.000 MW cut-off. In order to obtain the maximum filtration recovery (101±3%) the pretreatment of membranes with 0.1 M sodium hydroxide and bidistilled water was necessary. Results on the effect of tiaprofenic acid, an antiinflammatory non steroidal agent, on glibenclamide and tolbutamide levels in maturity onset diabetic patients are reported. Neither pharmakokinetic parameters, nor the free fraction of sulphonylureas were significantly changed after tiaprofenic acid treatment, thus excluding the risk of pharmacological interactions.

INTRODUCTION

Glibenclamide and tolbutamide are very commonly used hypoglycaemic drugs, effective in non-insuline dependent (type II)

diabetes mellitus (1-4). The "adult onset" diabetic patients, particularly the elderly ones, are usually under different concomitant therapies, i.e. non steroideal antyinflammatory drugs, which may cause displacing of sulphonylureas from protein binding sites (5). One of the major risks occurring during the use of oral hypoglycaemic drugs is thus the possible prolonged hypoglycaemia which, sometimes, may be a relevant adverse reaction (6). The pharmacokinetic and pharmacodynamic behaviour and the study of pharmacological interactions of sulphonylureas are very useful, giving indications about possible undesired complications.

Several analytical approaches are available, at present, for the measurement of these drugs (7-15) and are generally employed to determine of the total circulating levels in pharmacokinetic studies. The determination of the "free" fraction, i.e. that able to cross cell membranes, bind to receptors and express the pharmacological activity, instead, has not been carried out so often (16-25).

Since Sophianopoulos et al. (26) verified its validity, ultrafiltration has been increasingly employed as an alternative technique to the traditional method of equilibrium dialysis for the assay of free drugs. Most of the studies on glibenclamide and tolbutamide protein binding utilize equilibrium dialysis tecnique with the labelled drug, while only few workers utilize the ultrafiltration tecnique followed by GC or HPLC determination.

In this study we have developed a rapid and reproducible ultrafiltration procedure to assess the serum concentration of

free tolbutamide and glibenclamide. We compared the free and total levels of these agents in type II diabetic patients and then, the influence of a non steroidal antiinflammatory drug (tiaprofenic acid) on the protein binding equilibrium of sulphonylureas was also evaluated.

EXPERIMENTAL

Reagents and Solutions

Standard sulphonylureas were obtained from Hoechst (Milano, Italy). Tiaprofenic acid was kindly provided by Roussell Maestretti (Milano, Italy). Dinitrofluorobenzene (DNFB), potassium dihydrogen phosphate, hydrochloric acid were from Merck (Darmstadt, F.R.G.). The solvents (Merck) were HPLC grade and water was always bi-distilled. The solutions were always filtered through a 0.45 μm filter (Millipore, Molsheim, France) before HPLC analysis.

Equipment

For the separation of serum protein-bound fraction from the free one, a disposable Centricon micropartition system and a YMT membrane with 30,000 MW cut-off (Amicon, Danvers, MA, USA) were used. Ultrafiltration was performed at 4 °C on a centrifuge with a fixed angle rotor.

HPLC analysis was performed on a Kontron (Zurich, CH) liquid chromatograph, assembled with two model 420 pumps, model

460 autosampler, model 325 plotter and detector Uvikon 735. All modules were controlled and programmed by a Data System 450. The separation was performed on a LiChrosorb RP18 cartdrige (Merk) (250 X 4.6 mm i.d., 7μm particle size). Solvents were evaporated in a vacuum centrifuge (Savant, Hicksville, USA).

Chromatographic conditions

Acetonitrile-0.01 M phosphate buffer, pH 3.5 (80:20,v:v) was used as the elution mixture; flow rate was 1 ml/min and the U.V. detector was set at 360 nm.

Standard solutions

Stock solutions of tolbutamide and glibenclamide (1 μg/ml) were prepared in methanol and stored at 4 °C for 1 week. Every day working solutions of appropriate concentration were prepared by diluting the stock solutions with water. DNFB solution (6 mg/ml) was prepared weekly in n-butyl acetate and stored at 4 °C in the dark.

Glibenclamide and tolbutamide assay

A previously published method (9) was modified for our purposes. Serum (1ml), 1M hydrochloric acid (0.2 ml) and internal standard (10 μg glibenclamide for patients under treatment with tolbutamide and 1 μg tolbutamide for subjets taking glibenclamide respectively) were gently shaken with 5 ml toluene for 15 min. After centrifugation (1500 g for 3 min) the organic layer was

transferred to a 12 ml conical glass tubes with screw caps with PTFE facing, and evaporated to dryness. After addition of 25 µl of the derivatizing agent (DNFB), the samples were heated for 30 min at 120 °C, dried again and redissolved in 50 µl of the mobile phase before HPLC injection.

Ultrafiltration procedure

Before ultrafiltration, YMT membranes were pre-treated centrifuging 0.2 ml of 0.1 M sodium hydroxide, followed by rinsing with bi-distilled water (0.5 ml). Serum (1 ml) was placed in the Centricon sample device, capped and centrifuged at 2500 g for 90 min at 4 °C. The ultrafiltrate (0.7 ml) was transferred to the test tube, the internal standard was added and the sample processed as described for the total drug assay.

Recovery and calibration curves

Calibration curves for total and the free assay were constructed by spicking with increasing amount of the drugs a control serum or an ultrafiltrate of control serum, respectively. Linearity of the assay was determined by plotting the sulphonylureas-internal standard peak area ratios against the drug concentration in the spiked serum or ultrafiltrates. Recovery of the drugs from the ultrafiltration procedure was evaluated from the comparison of peak areas in acidified water samples spiked before and after ultrafiltration.

Application

Free and total drug concentrations were evaluated at hourly intervals for one day in type II diabetic patients under long term treatement with tolbutamide (500 mg t.i.d.) or glibenclamide (5 mg b.i.d.). The same protocol on the same patients was repeated after 5 days of additional treatment with tiaprofenic acid (300 mg b.i.d.) in order to evaluate the possible displacing of sulphonylureas from binding proteins.

Statistic

Results given are means ± standard deviation. Student's t test was used in the evaluation of the differences between means.

RESULTS AND DISCUSSION

Tolbutamide and glibenclamide are highly protein-bound drugs (95-99%) in healthy subjects (4,18,24). In order to assay the free circulating levels, a very specific and sensible method is therefore required, especially for glibenclamide which maximum serum concentration rarely exceed 300 ng/ml following a therapeutic dose. Several methods utilize an acidic extraction with different solvents (chloroform, benzene, diethyl ether) followed by UV (7) or fluorescent (8) detection. All these methods resulted not suitable for our pourposes, soffering to some degree of the lack of specificity and giving very dirty "blanks" extracts. We adopted the method by Zecca et al. (9) but we used

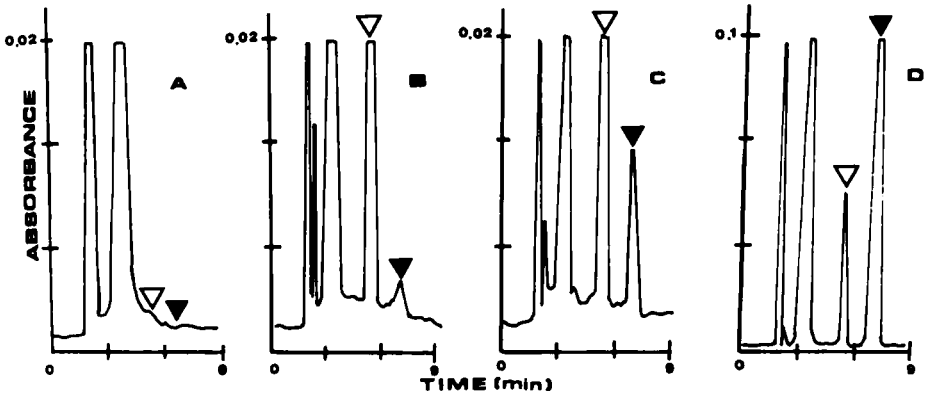

FIGURE 1: Chromatograms of A) control serum ultrafiltrate B) serum ultrafiltrate of a patient taking glibenclamide (free level 5 ng/ml) C) the same specimen analysed before ultrafiltration (glibenclamide concentration 68 ng/ml) D) serum ultrafiltrate from a patient taking tolbutamide (free level 0.5 µg/ml). In A) and B) 50/50 µl, in B) and in C) 25/50 µl were injected.

toluene, a more selective solvent, for extraction instead of chloroform, we reduced the quantity of derivatizing agent (25 µl), increasing on the contrary the concentration to obtain higher reaction yields. Fig. 1 shows the chromatograms obtained from the analysis of a control serum ultrafiltrate, of an ultrafiltrate serum from a patient under treatment with glibenclamide and one under treatment with tolbutamide. In the figure is also reported the serum containing glibenclamide assayed before ultrafiltation. No peaks were detected at the retention time of the two sulphonylureas. By the method reported here, the quantification of serum glibenclamide or tolbutamide can be carried out at a lowest limit of 2 ng/ml, being this the minimum concentration giving rise to a signal-to-noise ratio of 2. Extraction by the solvent gave

almost a complete recovery of the two drugs. Standard curves gave a linear response for both drugs (r=0.999). In Fig. 2 the pharmacokinetic profiles of a subject taking glibenclamide and one taking tolbutamide are shown. The total drug concentration-time curves were studied before and after 5 days treatment with tiaprofenic acid. No significant differences were found as far as drug levels and pharmacokinetic parameters are concerned.

For the free drug assay, initially we tried to ultrafilter the plasma samples on YMT membranes directly. The recovery obtained centrifuging water containing authentic standards was always < 50% for tolbutamide and < 20% for glibenclamide, indicating a relevant loss of drugs by binding to Centricon components. Repeated washing of the membranes by inverting the Centricon unit did not show any improvement in the recovery efficiency (+10% and +3% for tolbutamide and glibenclamide, respectively). A complete recovery of the two drugs (101$\pm$ 3%) was accomplished only by pre-centrifuging the YMT membranes with 0.1 M sodium hydroxide (0.2 ml) followed by a rinsing with bidistilled water (0.5 ml), therefore, this procedure was adopted before each sample assay.

Standard curves constructed on a control serum ultrafiltrate gave a linear response in the expected free-serum level range (0-5 μg/ml for tolbutamide and 0-500 ng/ml for glibenclamide). The equations of the lines calculated by linear regression analysis were Y = 0.195X - 0.006 (r=0.999) for tolbutamide and Y = 0.527X + 0.002 (r = 0.999) for glibenclamide (X expressed as μg/ml). The

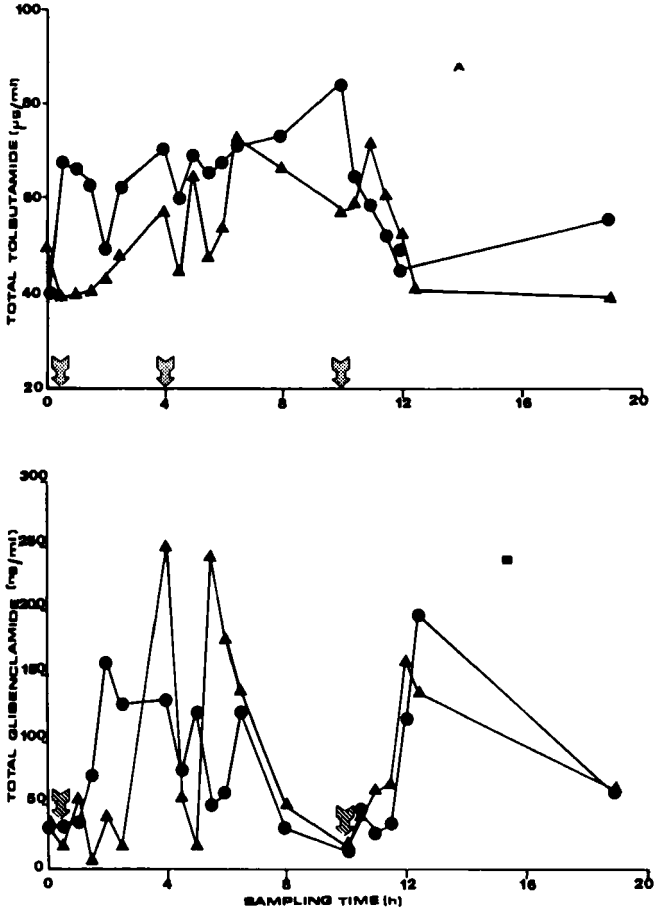

FIGURE 2: Total pharmacokinetic serum profiles of patients taking tolbutamide (A) or glibenclamide (B).
(▲) Before tiaprofenic acid treatment
(●) After 5-days treatment with tiaprofenic acid.
Arrows indicate time of administration of the two drugs.

ultrafiltration procedure showed good reproducibility. Water samples (6) of tolbutamide(1 µg/ml) or glibenclamide (25 ng/ml) ultrafiltered and analyzed in the same day gave a CV% of 2.5.

Evaluation of the possible displacing action of tiaprofenic acid treatment was done on selected serum samples taken at 0, 2.5, 5, 8, 10, 12.5, 19 h after sulphonylureas administration. Adir et al. (18) who considered the in vitro tolbutamide binding equilibrium, reported that the drug is about 95 % bound, this percentage being related to serum albumin and to the total drug concentration. Fernandez et al. (24) found that the percentage of free tolbutamide in serum of patients untreated with other drugs, was about 13%. In our study, the free levels of tolbutamide ranged from 0.8 to 9.3 µg/ml, with a mean value of 3.9 ± 4.0 (n=41) before tiaprofenic acid administration, while ranged from 0.6 to 5.5 µg/ml (2.9 ± 1.3) (mean ± SD, n=41) after the antiinflammatory treatment. The corrisponding mean free fractions were 4.6 ± 3.5 and 4.8 ± 2.7 % before and after the 5 days treatment, respectively.

In vivo protein binding behaviour of glibenclamide has not been extensively studied yet. Glibenclamide is a higly protein-bound drug (99%) in healthy subjects (4) and due to the very low circulating levels, some of the samples processed for the free fraction determination were under the detection limit of our method (2 ng/ml). In the samples reliable we found a mean value of 7.2 ± 6.5 and 7.7 ± 5.7 ng/ml (n=20) before and after tiaprofenic acid treatment, corresponding to a free fraction of 7.9 ± 6.2 and

7.3 ± 6.5%. We suppose that our slightly high free-serum glibenclamide levels, compared to those reported by Ferner et al.(4), are probably related to drug interaction with occasional drugs taken by our patients during the treatment (beta-receptor blocking drugs, antiarrhythmics etc.), to age (62±1.2 years) or to a probably slightly impaired renal function. However, no overall effects of tiaprofenic acid treatment on tolbutamide or glibenclamide free fraction in serum was detected, being the differences never significant. Fig. 3 shows the correlation between the free fraction and the total serum concentration of the two drugs. Although some authors already reported a strong correlation between the free fraction and the total concentration of sulphonylureas (18), we found only a moderate tendency (r= 0.18 and r=0.28 for tolbutamide and glibenclamide, respectively).

The described ultrafiltration technique resulted in a rapid and reliable method to determine the free serum levels of tolbutamide and glibenclamide in the therapeutic range and in presence of possible displacing agents that could enhance the free circulating concentrations, avoiding the much longer procedure of equilibrium dialysis. Moreover, Barre' et al. (27), for the determination of free valproic acid, din't find significant differences employing ultrafiltration or equilibrium dialysis, confirming the validity of ultrafiltration tecnique in respect to the considered reference method.

Different complications such as retinopathy or peripheral neuropathy have been frequently reported (4) as a consequence of

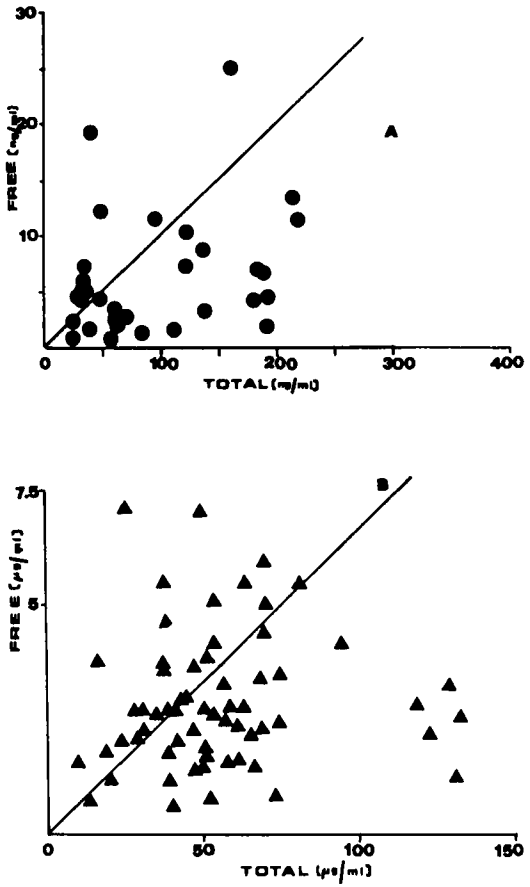

FIGURE 3: Free vs total glibenclamide and tolbutamide concentrations. The points reported for the two drugs are obtained both before than after the treatment with tiaprofenic acid.

an abnormal hypoglycaemic effect, produced by agents causing displacing of glibenclamide from protein binding sites. In these cases our simple and very accessible HPLC method may be very useful for physicians, being able to detect the increased glibenclamide free-serum levels and to monitor any possible drug

interaction. Tiaprofenic acid did not seem to modify either glibenclamide and tolbutamide serum levels, or pharmacokinetic parameters, thus excluding the risk of hypoglycaemia due to interaction with this pharmacological agent. However, our results can not be generalized for other sulphonylureas or other antiinflammatory drugs. Thus, given that the free drug represents the biological active fraction, it is desirable that the same kind of interaction is systematically explored.

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