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SIMPLE, RAPID AND MICRO HIGH-PRESSURE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF TOLBUTAMIDE AND CARBOXY TOLBUTAMIDE IN PLASMA

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SUMMARY

A rapid high-pressure liquid chromatographic (HPLC) assay is described for the quantitative analysis of tolbutamide and its major metabolite, carboxy tolbutamide, in plasma. An aliquot (25—100 μl) of plasma was prepared for chromatography by deproteinization as follows. One volume of plasma and 2.5 volumes of acetonitrile were vortex mixed for a few seconds and then centrifuged for approx. 1 min. A 50-μl sample of the clear supernatant was injected into the chromatograph. A μBondapak C_{18} reversed-phase column was used with a mobile phase of acetonitrile—0.05% phosphoric acid (45:55) at a flow-rate of 1.5 ml/min. The column effluent was monitored by a variable-wavelength UV detector set at 200 nm. Tolbutamide and its metabolite had retention times of 5.75 and 3.25 min, respectively. The procedure yields reproducible results with sensitivity adequate for routine clinical monitoring of plasma levels or for single-dose pharmacokinetic studies. A number of commonly used drugs do not interfere with the method. A single plasma sample can be analyzed in approx. 9 or 10 min.

INTRODUCTION

Tolbutamide is a sulfonylurea that is orally effective as a hypoglycemic agent and has found wide application for the treatment of diabetes mellitus of the maturity-onset type. It has been suggested that therapeutic plasma concentrations of tolbutamide lie in the range of 53—96 μg/ml [1]. The chronic nature of diabetes usually means that tolbutamide may need to be administered over long periods to a patient population comprised of middle-aged or elderly people in whom progressive physiological changes are occurring which could result in clinically important alterations in drug disposition or intrinsic responses. It is usually recommended that patients of advanced age begin ther-

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apy with about half the usual daily dose of tolbutamide because some such individuals are very responsive to sulfonylureas and may develop severe hypoglycemia after usual doses [2]. Therefore, it may be prudent to monitor the plasma level of tolbutamide occasionally in elderly patients who receive the drug over a prolonged period, to ensure the suitability of the established dosage regimen. Riegelman and Sadee [3] have advocated the monitoring of plasma levels of tolbutamide when the patient does not appear to have achieved the expected therapeutic response.

A number of methods based on absorption in the ultraviolet [4, 5] or visible [6—8] regions have been reported over the last twenty years for the quantitative determination of tolbutamide in biological fluids. However, those procedures suffer from disadvantages which include lack of adequate sensitivity and specificity, and laborious work-up procedures. Gas chromatographic procedures which have been described [8—12] overcome some of the disadvantages of the spectrophotometric methods, but the analysis time is greatly lengthened by the necessity to derivatize the tolbutamide molecule prior to gas chromatography. A report of an HPLC method for quantitative analysis of tolbutamide in 1-ml aliquots of plasma has recently appeared in the literature [13]. Sample preparation prior to chromatography required lengthy solvent extraction and evaporation steps.

The purpose of the present paper is to report a new HPLC method for the simultaneous quantitative analysis of tolbutamide and its carboxy metabolite in plasma. The method described involves an extremely simple sample preparation followed by HPLC analysis on a reversed-phase column with UV detection of the compounds in the column effluent. The assay is fast and sensitive, and only very small plasma samples are required.

EXPERIMENTAL

Reagents and standards

Tolbutamide and 1-butyl-3-(*p*-carboxyphenylsulfonyl)-urea (hereafter referred to as carboxy tolbutamide) were kindly supplied by Upjohn (Kalamazoo, Mich., U.S.A.). Standard solutions of these compounds were made up in methanol and stored at —20° when not in use. Glass-distilled methanol and acetonitrile were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and phosphoric acid was obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.).

Other drug substances which were tested for potential interference of the assay had, in most cases, been donated by pharmaceutical manufacturing companies. Solutions of these compounds in methanol or distilled water were prepared and stored at —20° when not in use.

A 0.05% solution of phosphoric acid in distilled water (final pH 2.6) was prepared and passed through a 0.45 μm membrane filter for subsequent use in the preparation of the HPLC mobile phase.

HPLC instrumentation and conditions

A model M-6000A pump was used to deliver the mobile phase to a model U6K injection loop and a 30-cm μBondapak C_{18} reversed-phase column (par-

Preliminary study in a rabbit

Tolbutamide (40 mg in 1.6 ml of 95% ethanol) was infused over 1 min into the medial vein of one ear of a 4-kg albino rabbit. Blood samples (0.5 ml) were collected from the marginal vein of the contralateral ear prior to drug administration and at intervals for 5 h thereafter. Blood samples were placed in heparinized tubes which were centrifuged immediately to obtain plasma. The separated plasma samples were stored at −20° until the time of analysis.

RESULTS AND DISCUSSION

Tolbutamide is metabolized in humans by oxidation to carboxy tolbutamide and this metabolite accounts for the majority (about 75%) of the urinary recovery of a dose of the drug [2]. Although this metabolite is reported to be devoid of hypoglycemic activity [14] it may be important from a toxicological standpoint. In addition, in some pharmacokinetic studies it may be desirable to study the time course of formation and removal of the metabolite. Therefore, the tolbutamide assay described in this paper has been designed so that it also accommodates quantitation of the major metabolic transformation product of tolbutamide.

The UV absorption spectrum of tolbutamide dissolved in the HPLC mobile phase is shown in Fig. 1. The earlier reported UV methods for quantitative analysis of tolbutamide [4, 5] made use of the peak at approx. 228—230 nm. Under the conditions employed in the present work the absorbance at 200 nm was approx. 2.5 times greater than the absorbance at 230 nm. As a result the lower wavelength (200 nm) was chosen for the monitoring of the HPLC column effluent by the UV detector although 230 nm could also be used.

Chromatograms resulting from the acetonitrile treatment and HPLC of blank human plasma together with similarly treated plasma which had been previously spiked with tolbutamide and carboxy tolbutamide are shown in Fig. 2. Tolbutamide eluted from the HPLC system after 5.75 min and was well resolved from its more polar carboxy metabolite which had a retention time of 3.25 min. No interference in blank plasma was observed at the retention time of tolbutamide for this particular sample of plasma or for plasma collected from eight other individual patients who were receiving other drugs. However, in some of the samples of plasma a small peak occurred which had a similar retention time to that of carboxy tolbutamide. The maximal contribution of this interfering peak observed in any of the various batches of plasma analyzed was of the order of 0.5 μg/ml.

Data used to establish the standard curves for tolbutamide and its metabolite are summarized in Tables I and II, respectively. Least squares linear regression analysis was carried out for each of the standard curves and the resulting coefficients of determination (r^2) were 0.9998 and 0.9996 for tolbutamide and carboxy tolbutamide, respectively. These high values of r^2 together with the constancy of the response factors (peak height divided by concentration) over the concentration ranges studied indicate good linearity for the standard curves. The percentage recovery from plasma for tolbutamide, compared to an aqueous solution, was 97 and 99% at plasma concentrations of 50 and 150 μg/ml, respectively. For carboxy tolbutamide the percentage

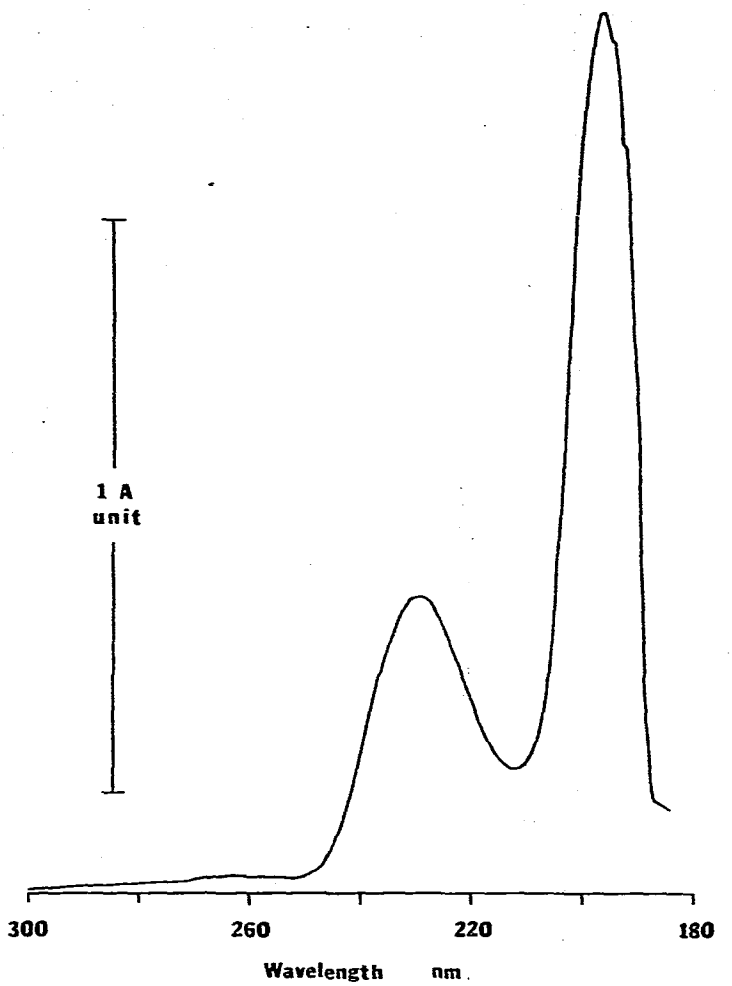

Fig. 1. UV absorption spectrum of a 1 mg% solution of tolbutamide in acetonitrile—0.05% phosphoric acid (45:55). The spectrum was recorded using a Model 200 Perkin-Elmer UV—visible recording spectrophotometer.

recovery from plasma, determined in a similar manner, was 87 and 89% at plasma concentrations of 25 and 75 μg/ml, respectively.

The within-day precision of the method was good, as assessed by conducting replicate (N = 10) analyses of the same spiked plasma sample (50 μg/ml of tolbutamide and 25 μg/ml of carboxy tolbutamide). The coefficient of variation for tolbutamide was 1.17%, while for carboxy tolbutamide the corresponding value was 2.32%. The coefficients of variation for the analysis of the same plasma sample on five days over a period of one week were 1.16% and 4.01% for tolbutamide and its metabolite, respectively. Although these data suggest that the day-to-day reproducibility of the method was good it may be prudent to include at least one standard sample on those days when patient plasma samples are being analyzed.

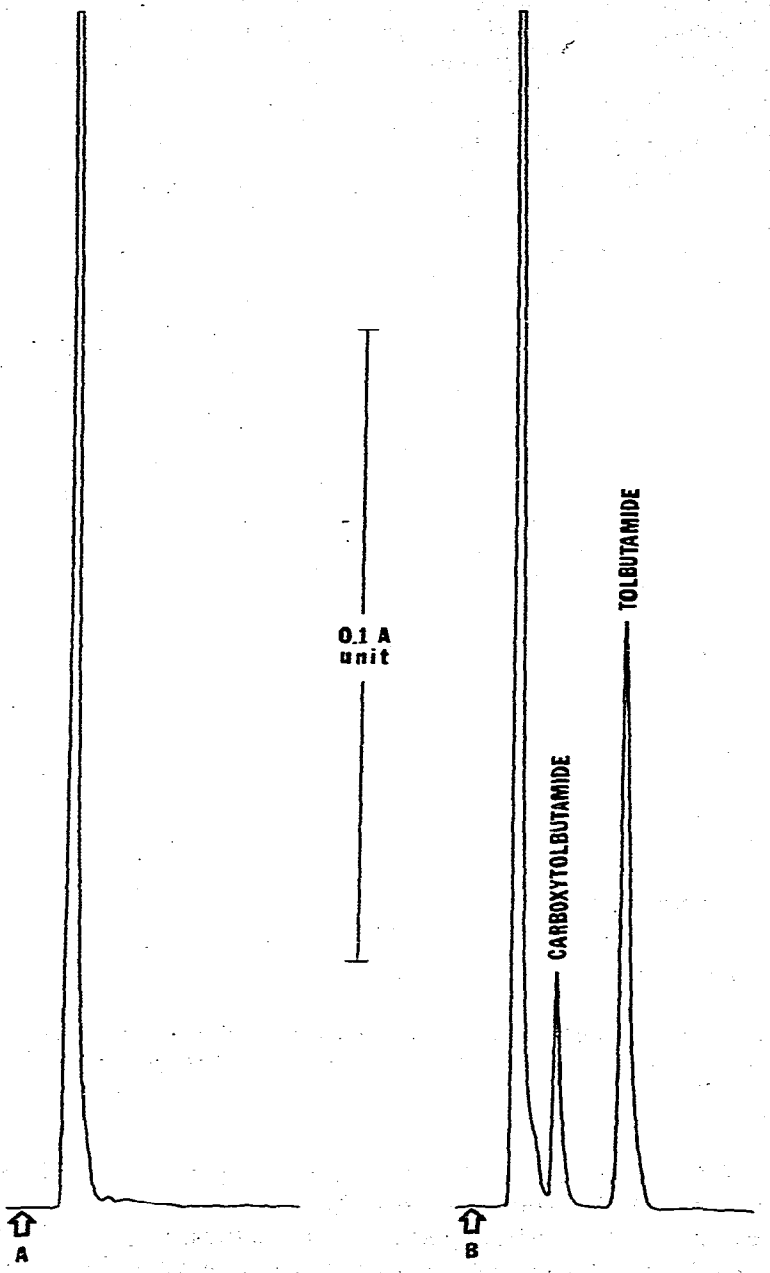

Fig. 2. Chromatograms of acetonitrile-treated blank human plasma (A) and similarly treated plasma which had been spiked with carboxy tolbutamide (25 μg/ml) and tolbutamide (50 μg/ml) (B). The arrow marks the point of injection.

TABLE I

STANDARD CURVE FOR TOLBUTAMIDE IN PLASMA

Linear regression equation: $y = 2.2909x + 1.7744$, $r = 0.9999$. One measurement was performed at each concentration.

Tolbutamide conc. in plasma (μg/ml)	Tolbutamide peak height*	Response factor**
5	11	2.21
10	23	2.28
25	57	2.29
50	119	2.38
100	237	2.37
200	457	2.28
300	689	2.30

*Peak height (mm) when detector sensitivity corresponds to 0.2 a.u.f.s.
**Peak height divided by tolbutamide concentration.

TABLE II

STANDARD CURVE FOR CARBOXY TOLBUTAMIDE IN PLASMA

Linear regression equation: $y = 1.9800x + 1.4117$, $r = 0.9998$. One measurement was performed at each concentration.

Carboxy tolbutamide con. in plasma (μg/ml)	Carboxy tolbutamide peak height*	Response factor**
2.5	5	1.93
5	10	1.99
12.5	26	2.04
25	54	2.15
50	103	2.07
100	197	1.97
150	299	1.99

*Peak height (mm) when detector sensitivity corresponds to 0.2 a.u.f.s.
**Peak height divided by carboxy tolbutamide concentration.

The addition of 2.5 volumes of acetonitrile to 1 volume of plasma followed by very brief (about 10 sec) vortex mixing results in deproteinization of the plasma. A short period of centrifugation readily separates the denatured proteins from the supernatant, which consist of plasma water and acetonitrile. Centrifugation causes the protein material to form a solid cake at the base of the culture tube and permits the supernatant to be decanted to another tube, although this latter step is not necessary. This method of preparing plasma samples for HPLC analysis is extremely simple and fast and has been used successfully in this laboratory for the analysis of a large number of other

compounds, including sulfisoxazole [15], creatinine [16], griseofulvin [17], procainamide and N-acetylprocainamide [18], theophylline [19], salicylates, and furosemide. Over a period of approx. one year many hundreds of deproteinized plasma samples have been injected into various types of HPLC columns (ion-exchange, reversed-phase, etc.) without any apparent alteration in column performance having occurred with time. No pre-columns or other special column care procedures (other than those recommended by the column suppliers) have been used during that time interval.

Different methods of deproteinizing plasma samples prior to the HPLC analysis of other drug molecules have been reported [20, 21] but the other procedures do not appear to offer simplicity and rapidity comparable with the acetonitrile deproteinization method.

It should be pointed out that a volume of plasma smaller than 100 μl can also be satisfactorily analyzed for tolbutamide and carboxy tolbutamide using the method described in this paper. The addition of 2.5 volumes of acetonitrile to 25-μl and 100-μl aliquots of the same spiked plasma sample, followed by vortexing, centrifugation, and injection of 50μl of the supernatant into the HPLC, yielded the same answer for the tolbutamide and metabolite concentration in the plasma sample. The ability to use such small plasma volumes is a great advantage in clinical situations, where capillary blood samples may be used, or for pharmacokinetic studies in small laboratory animals.

A study was made of the retention times of other sulfonylurea oral hypoglycemic drugs using the HPLC conditions described above. It was found that acetohexamide and tolazamide had almost identical retention times to that of tolbutamide, but chlorpropamide, with a retention time of 4.6 min, was resolved from the tolbutamide peak. The similarity of retention times for the four anti-diabetic drugs should in no way limit the usefulness of the tolbutamide assay since it would be very rarely that any one patient would concurrently receive more than one of the agents. It is anticipated, therefore, that tolazamide, acetohexamide, or chlorpropamide plasma concentrations could be determined using the method described here for tolbutamide, provided that the patient concerned was only receiving one of the drugs.

A large number of other drugs and drug metabolites were tested for potential interference of the assay by injecting stock solutions of the compounds into the HPLC. The compounds tested in this regard were as follows: acetaminophen, amethopterin, ampicillin, aspirin, caffeine, chloramphenicol, chlordiazepoxide, chlorpromazine, chlorthalidone, ephedrine, fluphenazine, glycinexylidide, lignocaine, methaqualone, monoethylglycinexylidide, N-acetylprocainamide, perphenazine, phenacetin, phenobarbitone, phenytoin, procainamide, prochlorperazine, salicylic acid, sulphisoxazole, tetracycline, theobromine, theophylline, triflupromazine, and trimeprazine. Methaqualone was the only compound which had a similar retention time to that of tolbutamide, although it should be noted that some of the other compounds tested would interfere with the analysis of carboxy tolbutamide. Plasma collected from eight patients who were receiving other drug therapy, which included ampicillin, chlorthalidone, digoxin, frusemide, methyl dopa, prednisone, and terbutaline, showed no interference at the retention time of tolbutamide and

only slight interference in some samples at the retention time of carboxy tolbutamide.

Fig. 3 shows chromatograms of acetonitrile-treated blank rabbit plasma and similarly treated plasma collected from a rabbit after the intravenous admini-

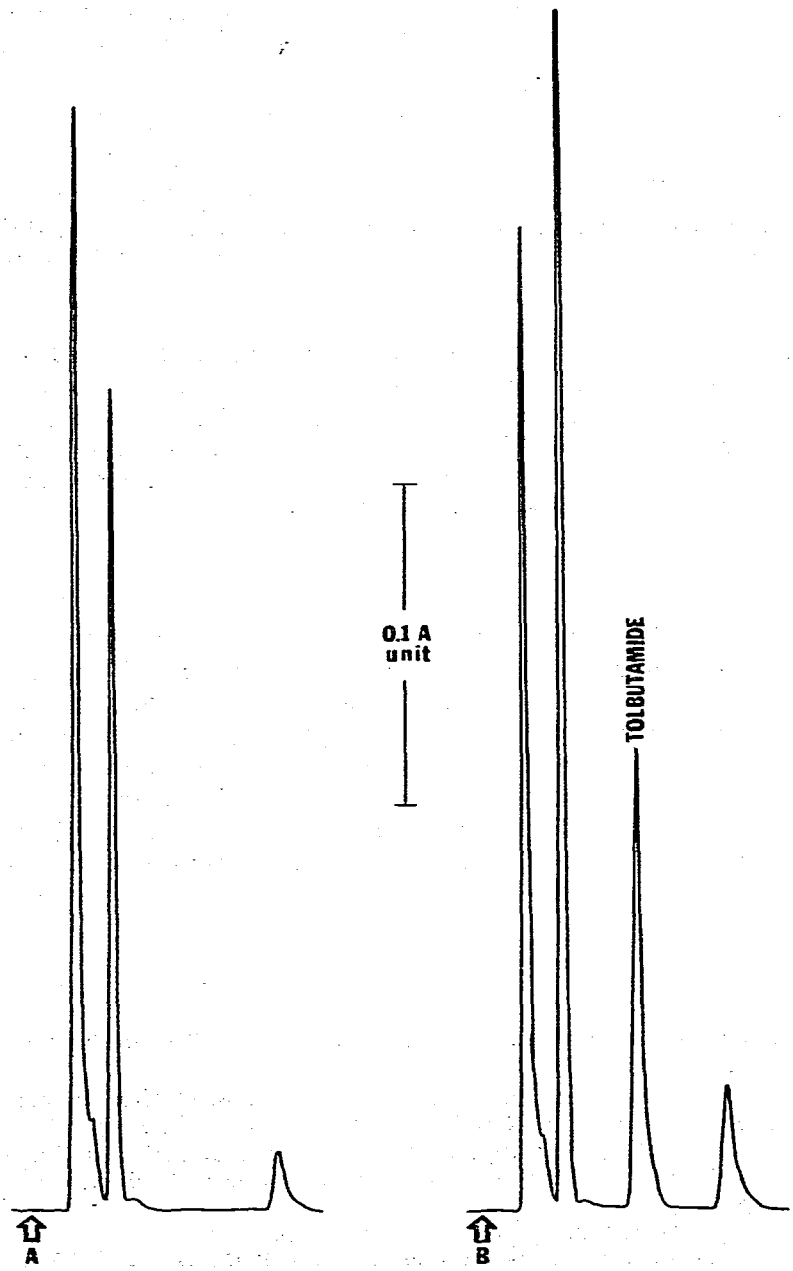

Fig. 3. Chromatograms of acetonitrile treated pre-dose (blank) rabbit plasma (A) and similarly treated plasma which was collected from the rabbit after intravenous administration of tolbutamide (B). Tolbutamide concentration in sample B was 78.6 μg/ml. The arrow marks the point of injection.

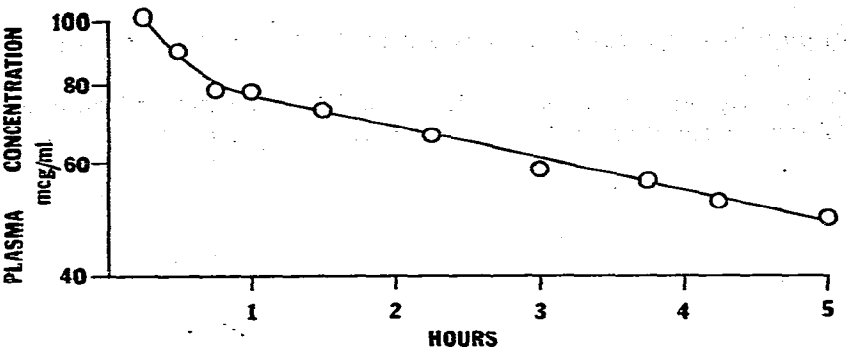

Fig. 4. Time course of tolbutamide plasma concentrations in a 4-kg rabbit following the intravenous injection of 40 mg of tolbutamide. The line represents a fit by eye to the data.

stration of tolbutamide. As can be seen from the analysis of the pre-dose (blank) plasma sample, no endogenous compounds eluted with a similar retention time to that of tolbutamide, but an interfering peak did elute near carboxy tolbutamide and made it impossible to quantitate that metabolite in rabbit plasma. The time course of tolbutamide plasma concentrations in the rabbit following intravenous administration is shown in Fig. 4.

CONCLUSIONS

The method described in this paper for the simultaneous determination of tolbutamide and carboxy tolbutamide in plasma is simple and rapid and requires only a micro volume of plasma. The total analysis time per sample is of the order of 9—10 min and no evaporation or derivatization steps are required Plasma concentrations of tolbutamide as low as 0.5 μg/ml could be quantitated, if necessary, although it should be noted that the precision of the method at that concentration has not been established. It is concluded that the analytical procedure may be valuable for the routine monitoring of plasma concentrations in patients receiving the drug and for pharmacokinetic studies in humans and animals.

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