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HIGH-PERFORMANCE LIQUID COLUMN AND THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF HUMAN SERUM GLIBENCLAMIDE AT THERAPEUTIC LEVELS

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SUMMARY

For glibenclamide bioavailability studies in serum, high-performance liquid column and thin-layer chromatographic methods were introduced. Both methods are specific, accurate and sensitive with detection limits of at least 5 ng of glibenclamide per ml of serum. Detection is performed in the ultraviolet at wavelengths of 200 nm for liquid chromatography or 300 nm for thin-layer chromatography.

Serum levels determined by either method correlated well with those determined by an already existing radioimmunoassay. Some pharmacokinetic data were computed using a one-compartment open model.

INTRODUCTION

Glibenclamide (HB 419)* (Fig. 1) is a highly potent antidiabetic agent. For pharmacokinetic purposes, especially for bioavailability studies of various preparations, analytical methods are required. They have to be practicable, specific, accurate and sensitive, allowing assaying at the low ng/ml of serum level.

Previously published methods employed radioimmunoassay [1—3], gas chromatography [4], polarography [5], colorimetry [6], fluorimetry [6, 7], and, more recently, high-performance liquid chromatography (HPLC) [8, 9]. Polarography, colorimetry and fluorimetry were not specific. For radioimmunoassay cross-reactions with sulfonylureas and their metabolites are encountered. Gas chromatography required a derivatization step. Furthermore,

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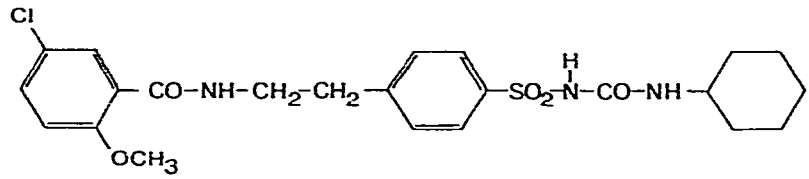


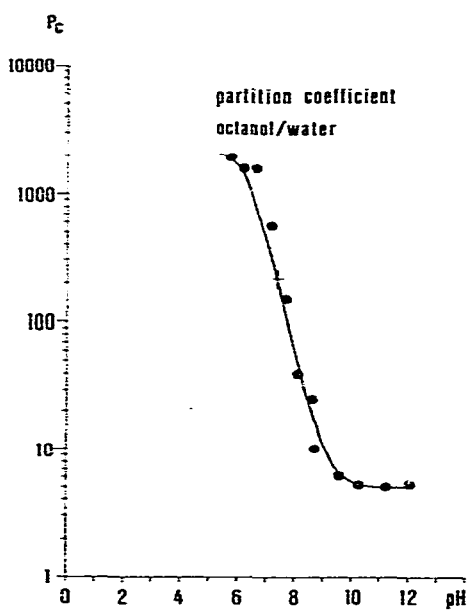
Fig. 1. Structure of glibenclamide.

the sensitivity of HPLC was not sufficient to follow a single oral treatment with a 2.5-mg tablet (cf. Fig. 10).

We therefore developed highly sensitive assays based on HPLC and thin-layer chromatography (TLC).

PHYSICOCHEMICAL PROPERTIES OF GLIBENCLAMIDE

Knowledge of the physicochemical properties of a compound is essential for obtaining optimal conditions for its extraction from serum and for choosing the most suitable conditions of measurement. The UV spectrum, solubility and partition of glibenclamide in ether/buffer have been published by Hajdú et al. [6]. Furthermore, we studied partition in octanol/Britton-Robinson buffer in the pH range 5—12. From these data, partition coefficients for the free acid and for the salt of ca. 2300 and 5.0 ± 0.3, respectively, were calculated (Fig. 2). The pK_a = 6.15 was determined using an extrapolation procedure [10] (Fig. 3) following tritrations in various methanolic solutions. This value equals the pK_a = 6.4 ± 0.2 resulting from the partition study.



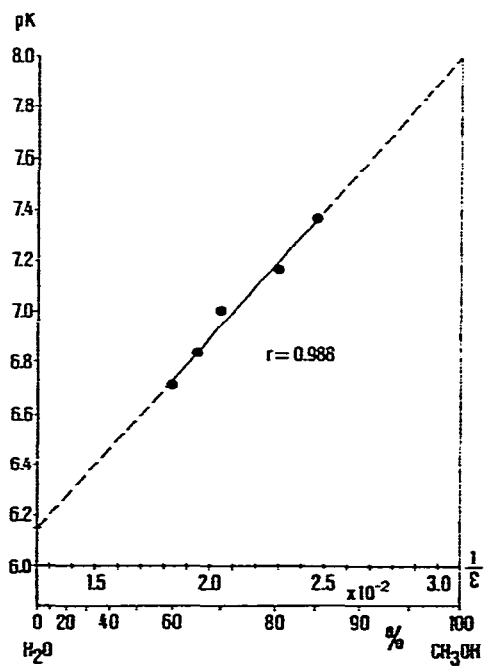


Fig. 2. Partition coefficient (octanol/water) of glibenclamide.

Fig. 3. Determination of pK_a following titration in methanolic solutions and extrapolation of pK_a (ϵ) to pK_a in aqueous solution.

EXPERIMENTAL

HPLC analysis

Reagents. The reagents used were 0.1 *N* hydrochloric acid AR, 5 *N* hydrochloric acid AR, 0.1 *N* sodium hydroxide AR, 1/15 *M* phosphate buffer solution (pH 7), diethyl ether AR, acetonitrile (HPLC grade S, Rathburn Chemicals, Walkerburn, Great Britain), the 4-methylcyclohexyl analogue of glibenclamide [N-(4-(β-5-chloro-2-methoxybenzamido-ethyl)benzenesulfonyl)-N′-(4-methylcyclohexyl)-urea] as internal standard (20 μg/ml in methanol). The mobile phase was acetonitrile—1/15 *M* phosphate buffer pH 7 (2:5, v/v).

Apparatus. The chromatograph consisted of a Waters M6000A pump with a Rheodyne 7100 injection port (100-μl sample loop) and a Schoeffel SF 770 spectrophotometer, equipped with a 10 cm × 4.6 mm I.D. column and a 3 cm × 4.6 mm I.D. pre-column filled with Spherisorb-ODS 5 μm (Brownlee MPLC system).

For sample clean-up a Vortex mixer, glass-stoppered tubes (ca. 12 ml capacity) and conical glass-stoppered tubes (ca. 8 ml capacity) were used.

Procedure. In a glass-stoppered tube, 1 ml of serum was treated with 0.2 μg of internal standard (10 μl) and 0.1 ml of 1 *N* hydrochloric acid. The serum was extracted for 20 sec with 5 ml of diethyl ether on a Vortex mixer; the phases were then separated by centrifugation (5 min) and 4.5 ml of the organic phase were transferred into a conical tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 100 μl of the mobile phase and 75 μl were injected into the chromatograph. The compound was detected at 200 nm (Fig. 4). At a flow-rate of 2 ml/min the retention times (t_R) found and k' values calculated were t_R = 4.0 min (k' = 7.9) for glibenclamide, and t_R = 7.6 min (k' = 15.9) for the internal standard (cf. Fig. 5). Quantitation was based on the peak height ratio of glibenclamide/internal standard.

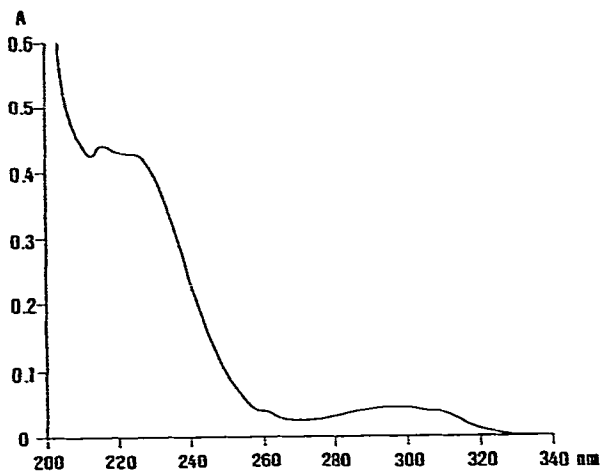


Fig. 4. UV spectrum of glibenclamide in buffered aqueous solution, 10 μg/ml of buffer (pH 6).

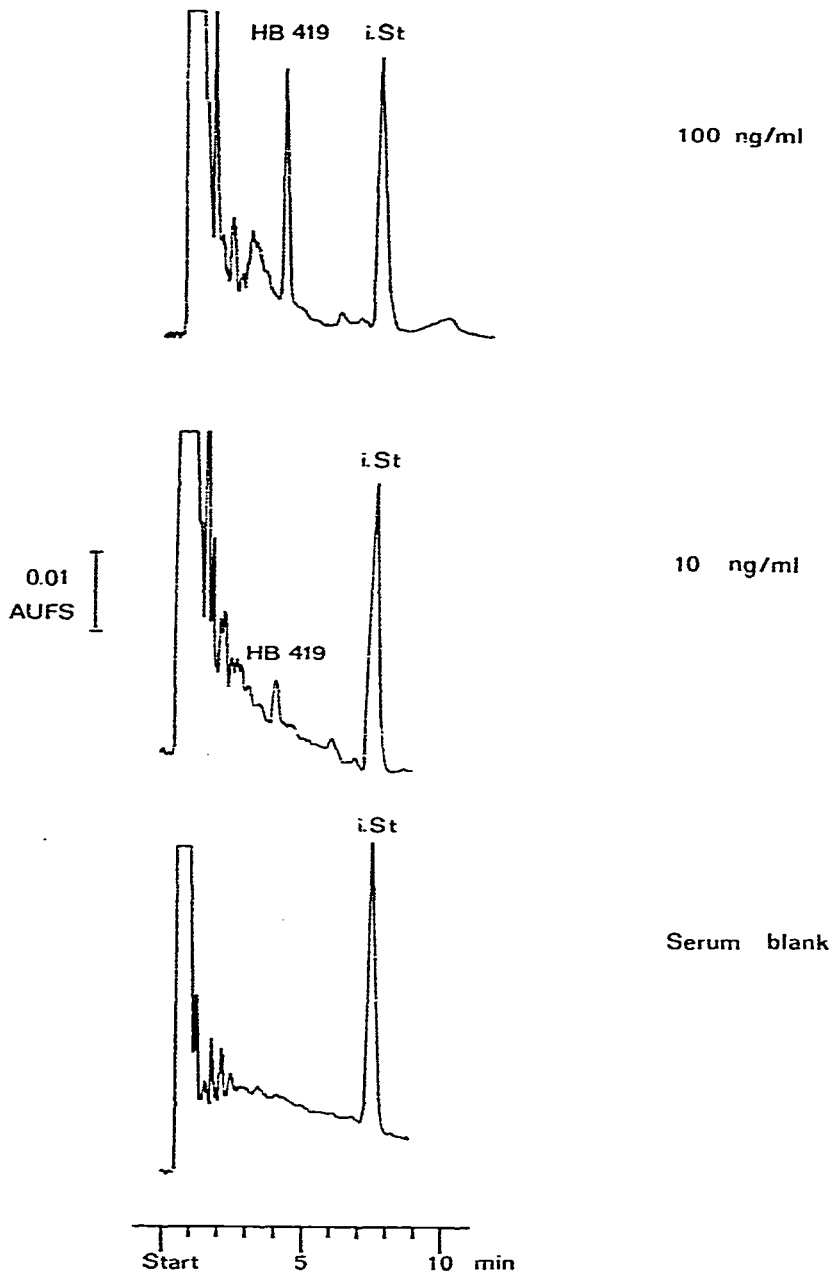


Fig. 5. Determination of glibenclamide (HB 419) in serum by HPLC, 10 and 100 ng/ml serum, compared with a serum blank.

TLC analysis

Reagents. The reagents used were buffer pH 4.62 (No. 36050, Riedel-de Haën, Seelze-Hannover, G.F.R.), diethyl ether AR, chloroform AR freshly distilled, methanol AR. The solvent system was chloroform—methanol—concentrated ammonia (15:3:0.2).

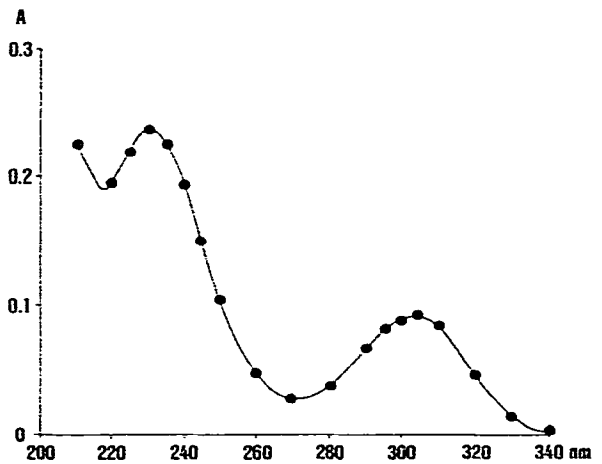
Equipment. A Zeiss KM3 chromatogram spectrophotometer with micro-optics and Servogor® 210 (Metrawatt) recorder were used. Separation was performed on F_{254} HPTLC plates (No. 5642, E. Merck, Darmstadt, G.F.R.) in a Camag twin-trough HPTLC chamber 20 cm × 10 cm (No. 25254).

For sample clean-up and spotting, a Vortex mixer, a centrifuge, glass-stoppered tubes (ca. 8 ml), conical glass-stoppered tubes (ca. 8 ml) and a Desaga Autospotter were used.

Procedure. In a glass-stoppered tube, 0.5 ml of serum was treated with 0.5 ml of buffer (pH 4.62). The serum was extracted with 5 ml of diethyl ether for 30 sec on a Vortex mixer, the phases were separated by centrifugation (5 min), and 4 ml of the organic phase were transferred into a conical tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 100 µl of chloroform. Using the Desaga Autospotter*, 75 µl were transferred on to the HPTLC plate as a series of consecutive droplets of ca. 100 nl volume each. Since each of these drops evaporated before the next one fell, narrow spots were obtained suitable for a high-performance TLC procedure.

The twin-trough HPTLC developing chamber contained 10 ml of the solvent in one compartment. The plate was developed over a distance of 6 cm in the dark and without previous saturation. The R_F value of glibenclamide was 0.5.

Measurements of the plates were carried out in the reflectance mode in the direction of the solvent flow with an effective slit (micro-optics) of 4.5 mm × 0.15 mm at a wavelength of 300 nm** (Fig. 6), scanning speed 50 mm/min and paper speed 240 mm/min. Peak areas of glibenclamide were evaluated and quantified by means of a calibration graph based on parallel analysis of known serum standards on the same plate.



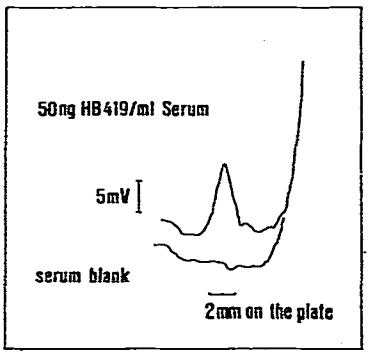


Fig. 6. In situ UV spectrum of glibenclamide on an HPTLC plate, 0.5 µg per spot.

Fig. 7. Determination of glibenclamide (HB 419) in serum by HPTLC, 50 ng/ml of serum, compared with a serum blank.

*Modified version, Tygon tubes of larger diameter [Technicon, flow-rated, code 116-0549-09 (white)] and 60 cm long Teflon tubes were used.

**Peak heights at 230 nm are considerably greater. However, as we experienced, interference from biological matter then induces higher variations of the results.

RESULTS

For HPLC and TLC the compound was admixed independently to blank serum in, respectively, seven and eight concentrations covering the range 2—500 ng/ml serum. Each sample was split into six portions, so that six equal series were formed. Each series was then analyzed in turn so that a total of six independent analytical results were available for each concentration.

Quality criteria

In correspondence with the recommendations of IUPAC [11] and IFCC [12], quality criteria of an analytical method are precision, accuracy, sensitivity and specificity. The corresponding parameters are derived from the analytical results given in Table I.

TABLE I

GLIBENCLAMIDE DETERMINATION BY HPLC AND TLC, RECOVERY AND ASSAY PRECISION

n = 6 determinations.

Added (ng/ml)	Found (ng/ml)	
	HPLC	TLC
500	501 ± 21	498 ± 13
250	247 ± 13	—
200	—	199 ± 5.2
100	98 ± 9.2	100 ± 3.3
50	49 ± 4.0	51 ± 2.5
25	23 ± 2.3	—
20	—	24 ± 2.3
10	11 ± 1.1	10 ± 1.8
5	6 ± 3.1	4 ± 1.0
2	—	2 ± 1.6
Blank	0	0
Precision*	± (3.9% ± 2.5 ng/ml)	± (2.3% ± 1.3 ng/ml)
Accuracy**	—0.7 ± 1.7 ng/ml	+0.1 ± 1.8 ng/ml

*See text under Precision.
**See text under Accuracy.

Precision. The standard deviations (S.D.) of the results obtained from each sample were considered to be a measure of precision. Experience has shown that precision decreases with decreasing concentration. Considering this effect, we expressed the standard deviation of the method as a function of concentration: glibenclamide by HPLC, S.D. = 2.5 ng/ml +3.9% of concentration in ng/ml; glibenclamide by TLC, S.D. = 1.3 ng/ml +2.3% of concentration in ng/ml.

Accuracy. Accuracy was depicted by the deviation (bias) at the mean value of the results from the theoretical value. In the case of glibenclamide, average

accuracy was < 1 ng/ml for both analytical methods. Regression coefficients were better than 0.999.

Sensitivity. The limit of detection is considered to be a measure of sensitivity. Whereas the limit of detection in a purely qualitative chromatographic separation is defined by the signal-to-noise ratio, it is a function of the precision of a method in the case of a quantitative procedure. As the precision decreases with decreasing values, a threshold value is obtained whose standard deviation is so large that statistically significant differentiation from zero is no longer possible. This value is defined as detection limit (DL)

$$DL = t_{n,95\%} \cdot \sigma_{c \to 0}$$

which for $n = 6$ becomes

$$DL = 2.02 \cdot \sigma_{c \to 0}$$

Thus, for glibenclamide the limit of detection calculated from the precision of the method for values approximating zero was 5 ng/ml in the case of HPLC compared to 3 ng/ml in the case of TLC.

Specificity. Specificity can only be defined verbally: so far, neither metabolites nor other drugs interfering in the determination of glibenclamide are known. However, it should be pointed out that this statement can not be definitive and should be reconsidered whenever applying this method to other species or in the case of new, concurrent medication.

Comparison with radioimmunoassay

Accuracy as described above refers only to spiked samples. Accuracy in true samples, i.e. from human or animal trials, has to be proved by comparison with results obtained from identical samples using an independent method. Both the TLC and HPLC methods were therefore compared independently with the radioimmmunoassay method, which up to now served as the standard procedure in our company [13]. The results correlated well (cf. Figs. 8 and 9).

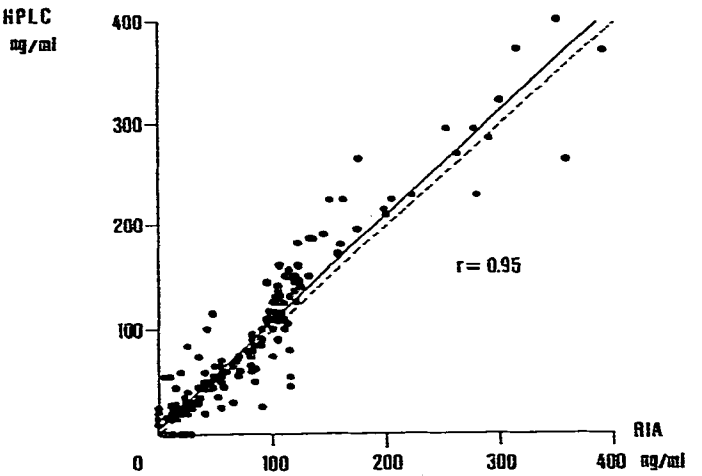


Fig. 8. Parallel analyses of 174 human serum samples by radioimmunoassay (RIA) and HPLC. HPLC: $\bar{c}$ = 88 ng/ml, S.D. = 23 ng/ml ≈ 27% of $\bar{c}$. RIA: $\bar{c}$ = 80 ng/ml, S.D. = 25 ng/ml ≈ 31% of $\bar{c}$. C_{HPLC} = (1.03 ± 0.03 · C_{RIA} + (5 ± 3) ng/ml. C_{RIA} = (0.88 ± 0.02) · C_{HPLC} + (3 ± 3) ng/ml.

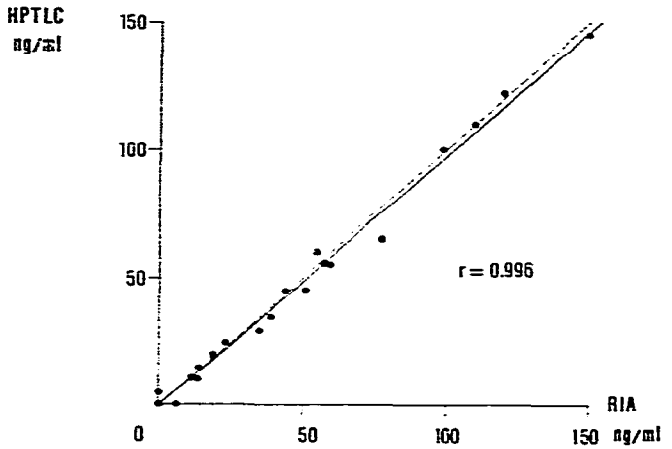


Fig. 9. Parallel analyses of 27 human serum samples by radioimmunoassay (RIA) and HPTLC. HPTLC: $\bar{c}$ = 36 ng/ml, S.D. = 3.7 ng/ml ≈ 10% of $\bar{c}$. RIA: $\bar{c}$ = 37 ng/ml, S.D. = 3.7 ng/ml ≈ 10% of $\bar{c}$. C_{HPTLC} = (0.98 ± 0.02) · C_{RIA} + (−0.6 ± 0.9) ng/ml. C_{RIA} = (1.02 ± 0.02) · C_{HPTLC} + (0.8 ± 1.0) ng/ml.

Pharmacokinetics

Glibenclamide was administered orally in doses of 2.5 mg to three healthy male volunteers (volunteer I, age 32 years, height 1.84 m, weight 89 kg; II, age 36 years, height 1.92 m, weight 69 kg; III, age 50 years, height 1.92 m, weight 80 kg)*. Serum levels were determined and the pharmacokinetic profiles were calculated based on the Bateman function. This is demonstrated by the serum kinetics obtained from the three volunteers in Fig. 10.

After a lag time of 1.8 ± 0.7 h, glibenclamide was absorbed with a half-life of 0.6 ± 0.2 h. Maximum serum levels of 123 ± 9 ng/ml serum were observed 3.2 ± 0.9 h post administration. The drug was eliminated with a half-life of 1.6 ± 0.3 h.

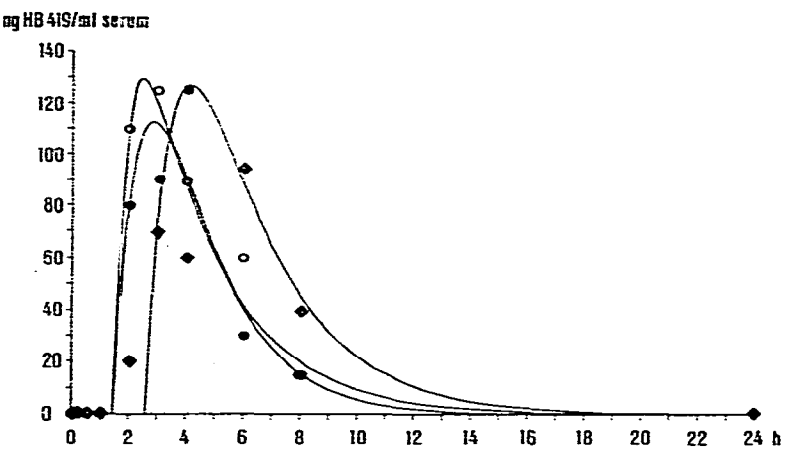


Fig. 10. Serum pharmacokinetics after oral application of 2.5 mg glibenclamide (HB 419) to volunteers I (•), II (◆) and III (○).

*These studies were performed by Drs. W. Rupp and M.J. Badian, Hoechst AG.

DISCUSSION

Both analytical methods, HPLC and TLC, were shown to be practicable and sensitive enough for routine determinations of glibenclamide in human serum. Therefore either of these methods may be employed whenever the use of the radioimmunoassay is not possible. Performance differences between both assays presented here are marginal.

Because of its flexibility, TLC should be preferred when small numbers of samples are to be analyzed. For larger series we prefer HPLC for which automated equipment has become more easily available. Since, meanwhile, TLC scanning also may be automated [14], the choice of the "appropriate" method should be based in any instance on a laboratory's equipment as well as experience.

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