Determination of Glibenclamide in Human Serum by HPLC

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Summary

The antidiabetic drug glibenclamide can be reliably quantitated in human serum with high performance liquid chromatography. The serum is buffered and extracted with toluene. The organic solvent is evaporated, the residue dissolved in the mobile phase and an aliquot sampled automatically and chromatographed. UV-detection at 229 m allows a lower limit of quantitation of 5 ng/ml. Precise handling of exact volumes facilitates external calibration. Statistical data for imprecision and inaccuracy are given and illustrate reliable quantification. Application of the method to experimental and clinical pharmacokinetic studies with specific problems is illustrated.

1 Introduction

Sulfonylurea drugs are widely used in the treatment of *diabetes mellitus*. Glibenclamide belongs to the improved second generation sulfonylureas with high potency. Whereas first generation drugs like tolbutamide are dosed daily to 500-1000 mg, glibenclamide is dosed as low as 1.75-3 mg per day. The resulting plasma concentrations are in the low nanogram region. To evaluate the pharmacokinetics, especially after single doses, highly specific, sensitive, and accurate analytical methods with high sample throughput are required.

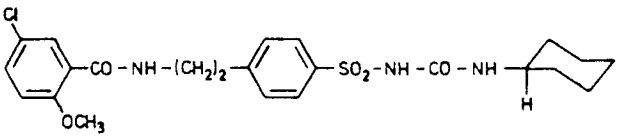

Figure 1

Chemical structure of glibenclamide.

In the past, ^{14}C-labelling [1,2], radioimmunoassay [3-9], colorimetry [10,11], and polarography [12] were used to quantify glibenclamide in body fluids. None of these methods were specific and strong interferences by metabolites were encountered. Published GC methods [13-17] have used time-consuming derivatizations. Only HPLC allows the automatized processing of numerous samples per time unit with simple sample-preparation. However, the previously published methods lack sensitivity, use gradients, have poor recovery, or were not applied to real samples. We describe a method which allows automatized processing of 60 samples per day with good accuracy and precision and high sensitivity. The method was applied to more than 1000 samples of different clinical studies.

2 Experimental

2.1 Materials

A pure standard of glibenclamide was provided by the British Pharmacopea Commission (BPCRS, Stanmore, UK). The purity, based on the BP 1980-procedure, was >99.5% by TLC. All other reagents, analytical or HPLC grade, were purchased from E. Merck (Darmstadt, FRG). Mobile phase components (water, acetonitrile, perchloric acid) were separately filtered prior to mixing, then degassed with helium after mixing.

2.2 Apparatus and Chromatographic Conditions

An automated high pressure liquid chromatograph consisting of modular components was used. Components were a LDC Constametric III solvent delivery pump, a Kratos SF 757 variable wavelength spectrophotometer operated at 229 nm, and a Waters model 710 B automatic sample processor. Detector output (peak area) was quantitated using a Shimadzu CR 1 B integrator. Data regression and concentration calculations were performed on a Commodore CBM 8032 Computer using the LAB CAL software program. The column was Shandon Hypersil ODS, 5 µm, 125 × 4 mm, packed by *M. Grom* (Ammerbuch, FRG). The mobile phase consisted of water (600 g), acetonitrile (320 g), and perchloric acid (5 g). The flow rate was 1.5 ml/min. All analyses were performed at room temperature.

2.3 Stock Solutions

Standard solutions of glibenclamide were prepared by dissolving 100.0 mg pure substance in 100 ml acetonitrile. Working solutions were prepared by appropriate dilution with acetonitrile, spiking solutions by further dilution with water. Solutions were stable for at least three months when stored at 4°C in the freezer.

Table 1

Results of the glibenclamide calibrations after 1/x-weighted regression.

Cal-No.	Concentration spiked in ng/ml					
	5.0	10.0	25.0	50.0	100	150
	Concentration found in ng/ml					
1	4.9	9.8	24.4	52.1	107	142
2	5.4	9.5	22.1	51.8	110	141
3	5.7	9.1	23.1	50.6	100	152
4	5.1	9.6	25.2	50.8	101	149
5	4.8	10.4	25.6	52.6	95	154
6	4.8	9.2	27.4	48.6	96	152
7	5.3	9.6	24.9	54.2	101	150
8	4.3	10.8	25.1	51.5	102	148
N	8	8	8	8	8	8
MEAN	5.0	9.8	24.7	51.5	102	149
SDEV	0.43	0.58	1.60	1.63	2.31	4.72
Accuracy	−0.2%	−2.0%	−1.2%	+3.0%	+2.0%	−0.7%
Precision	8.7%	5.9%	6.5%	3.2%	2.3%	3.2%
Mean Slope:		801 +/− 34				
Mean intercept:		0.43 +/− 0.2				
Mean R^2:		0.997 +/− 0.003				

2.4 Preparation of Samples

The sequence described has to be followed exactly, otherwise the results are not reproducible. 1.00 ml serum, 1.00 ml 2 M sodium dihydrogen phosphate, and 6.00 ml toluene were added to 10 ml round bottom culture tubes equipped with PTFE-lined screw-top caps. The samples were shaken in the vortex shaker for 60 seconds. After centrifuging at 5000 rpm, exactly 5.00 ml of the organic phase was transferred to a 10 ml tapered glass tube. The organic phase was evaporated under nitrogen on the water bath at 40°C. The tube walls were rinsed with dichloromethane after evaporation. The dry residue was redissolved in 200 µl mobile phase and 125 µl of the solution injected onto the chromatograph.

Table 2

Results of the recovery from serum.

Spiked concentration in ng/ml	Recovery from serum in %	Number of determinations
5.0	93	3
10.0	89	2
25.0	94	3
50.0	95	3
100	92	3
150	93	2

3 Results

3.1 Evaluation of the Method

The low serum levels of glibenclamide require sensitive detection. The native fluorescence at excitation wavelengths 313 nm, 229 nm, and 204 nm is too weak to be useful for low nanogram detection, even if the fluorometer is fitted with intense line-emitting lamps such as zinc, cadmium, or iodine lamps. The hydrodynamic voltammogram shows a high oxidation potential of 1.2–1.5 volts with poor response. The only practical detection principle is UV-detection. The UV-maximum at 310 nm has a low specific extinction coefficient. A second maximum at 229 nm is one order of magnitude more intense, the third maximum at 200 nm is the most intense but the least selective. In a precursor method the absorbance ratio at 200 and 207 nm was recorded, which equals the factor 2 in the chosen mobile phase. With selected, paired detectors and careful adjustment, this ratio can be used to control quantitation by measuring peak area or peak height at 200 nm.

Interferences at 200 nm were discovered by an enhanced ratio. In this case, the peak was quantitated at 207 nm. Though this procedure allows reliable quantitation, the instrumental and calculation expense is high. Detection at 229 nm has the advantage of higher selectivity; with state of the art detectors the sensitivity is sufficient for application to pharmacokinetic studies. From a lower limit of quantitation of 5 ng/ml serum (about 2.6 ng absolute) the method is linear to an upper limit of quantitation of 150 ng/ml serum with regression coefficient >0.995. **Table 1** shows the results of eight calibration sequences with spiked glibenclamide concentrations from 5 ng/ml to 150 ng/ml. The peak areas were processed by 1/x-weighting; from the

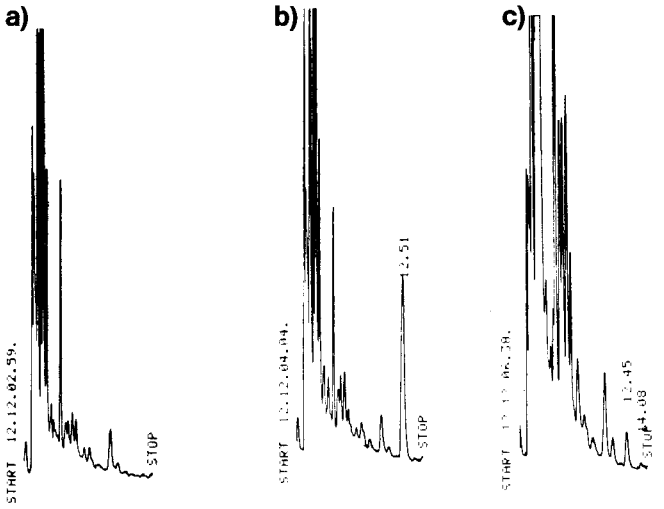

Figure 2

Typical chromatograms from a pharmacokinetic study with an administered single dose of 1.75 mg glibenclamide. a) Predose serum of a subject. b) Serum 45 minutes after administration; peak at 12.51 is about 62 ng/ml glibenclamide. c) Serum 8 hours after administration; peak at 12.45 is about 11 ng/ml glibenclamide.

weighted results the slope and intercept of the calibration curve wes determined. The recovery was checked between both limits of quantitation; from serum it is almost quantitative. **Table 2** presents the results.

3.2 Application to Human Studies

With this improved method more than 1000 samples from several different studies with different formulations have been measured. **Figure 2** shows typical chromatograms of real samples. In routine analysis several effects occurred, influencing the procedure of automated sampling. Depending on the individual subject, some late eluting peaks ($k' > 15$) were present in serum extracts, interfering or preventing quantitation in the next or a later run. This drawback can be overcome by matching the run time so that these peaks elute near the solvent front. The real run time then increases to about 25 minutes. Toluene has to be removed very carefully, as it elutes shortly after the analyte. Another problem is the presence of a notorious interference near the glibenclamide peak in the serum of some subjects. About 5% of all subjects tested ($n = 120$) had this interference. The peak area was on the order of 5 to 20 ng of glibenclamide and was not influenced by drug or food intake. Some other authors [18,24] also report this interference, finding increased levels in elderly patients. Our subject pool comprises male and female healthy caucasians between 18 and 60 years, therefore our data may reflect a statistical value. In most cases the interference can be separated from the analyte to allow quantitation with reduced precision. If this is not possible, the peak area of the subject blank is subtracted from the peak areas of the other samples assuming a constant interference level, which seems reasonable according to our experience. The chemical structure of this interference is not known.

3.3 Statistical Evaluation of the Assay Quality

The unknown concentrations of the samples were quantitated by comparison with the peak areas of samples spiked with known concentrations. We used external calibration, as the tested substances tolbutamide, carbutamide, glibornuride, glipicide, and tolazamide were either not extracted or came within the biogenic interferences and the recovery from serum was almost quantitative.

At the limit of quantitation, the imprecision was 8.8% with an inaccuracy of −1.2%, at higher concentrations the imprecision reduces to less than 4% with a typical inaccuracy lower than 3%. The specificity of the assay was tested with nicotine, coffeine, salicyclic acid, and propranolol. Neither these drugs nor other antidiabetica interfere with the assay.

4 Conclusion

This paper describes a rapid and sensitive HPLC method for quantitation of glibenclamide in human serum. The analyte is extracted from buffered serum into organic solvent and the solvent evaporated under nitrogen on the water bath. The residue is dissolved in mobile phase and sampled automatically. The limit of quantitation is sufficient for essentially any type of basic or clinical pharmacokinetic study as well as drug monitoring purposes. The external calibration procedure gives precise, reproducible, and accurate results as demonstrated by statistical evaluation. Specific problems of routine analysis are several kinds of interferences, which vary between subjects. Methods to overcome these problems are described.

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References

[1] *W. Rupp, D. Christ,* and *W. Heftner,* Arzneim.-Forsch./Drug Res. **19** (1969) 1428.

[2] *W. Rupp, D. Christ,* and *W. Fülberth,* Arzneim.-Forsch./Drug Res. **21** (1972) 471.

[3] *H. H. Borchert, H. Müller,* and *S. Pfeifer,* Pharmazie **31** (1976) 45.

[4] *M. E. Royer, H. Ko, J. S. Evans,* and *K. J. Johnston,* Anal. Lett. **9** (1976) 629.

[5] *P. Glogner, N. Heni,* and *L. Nissen,* Arzneim.-Forsch./Drug Res. **27** (1977) 1703.

[6] *D. Castoldi* and *D. Tofanetti,* Clin. Chim. Acta **93** (1979) 195.

[7] *K. Kawashima, T. Kuzuya,* and *A. Matsada,* Diabetes **28** (1979) 221.

[8] *B. Sartor,* Diabetologia **16** (1980) 17.

[9] *G. Ayanoglu, P. U. Witte,* and *M. Badian,* Int. J. Clin. Pharmacol. Ther. Tox. **21** (1983) 479.

[10] *P. Haydu, K. F. Kohler, F. H. Schmid,* and *H. Spingler,* Arzneim.-Forsch./Drug Res. **19** (1969) 1381.

[11] *R. Becker,* Arzneim.-Forsch./Drug Res. **27** (1977) 102.

[12] *S. Silvestri,* Pharm. Acta Helv. **47** (1972) 209.

[13] *K. Sabih and K. Sabih,* J. Pharm. Sci. **59** (1970) 782.

[14] *D. L. Simmons, R. J. Ranz,* and *P. Picotte,* J. Chromatogr. **71** (1972) 421.

[15] *K. K. Midha, I. J. McGilveray,* and *C. Charette,* J. Pharm. Sci. **65** (1976) 576.

[16] *H. J. Schlicht, H. P. Gelbke,* and *G. Schmidt,* J. Chromatogr. **155** (1978) 178.

[17] *P. Hartvig, C. Fagerlund,* and *O. Gyllenhaal,* J. Chromatogr. **181** (1980) 17.

[18] *E. Wahlin-Boll* and *A. Melander,* J. Chromatogr. **164** (1979) 541.

[19] *W. J. Adams* and *D. S. Krueger,* J. Pharm. Sci. **68** (1979) 1138.

[20] *M. Remmler, C. Lindner,* and *J. Oldendorp,* Z. Anal. Chem. **301** (1980) 110.

[21] *G. Lindner, L. Herbertz,* and *H. Reinauer,* Lab. Med. **4** (1980) 34.

[22] *F. Besenfelder,* **4** (1981) 237.

[23] *M. Uihlein* and *N. Sistovaris,* J. Chromatogr. **227** (1982) 93.

[24] *H. Pötter, M. Hülm,* and *K. Richter,* J. Chromatogr. **273** (1983) 217.

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