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Wide concentration range investigation of recovery, precision and error structure in liquid chromatography

U. Schepers[a], J. Ermer[b], L. Preu[a], H. Wätzig[a,*]

[a] *Institute of Pharmaceutical Chemistry, TU Braunschweig, Beethovenstrasse 55, D-38106 Braunschweig, Germany*
[b] *Aventis, Global Analytical Development, Industrial Park Höchst, Building G875, 65926 Frankfurt am Main, Germany*

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Dedicated to Prof. Dr. S. Ebel on the occasion of his 70th birthday.

Abstract

Using a typical HPLC assay, the characteristics of recovery, system precision and repeatability were investigated over a wide concentration range. In the presence of a constant amount of typical tablet excipients, the antidiabetic drug glibenclamide was analyzed in the range from 0.24 to 0.005 mg/mL (18 concentration levels, 6 independent sample preparations each). On the basis of a typical concentration for an HPLC glibenclamide assay of 0.2 mg/mL, this corresponds to a relative amount of 120–0.025% label claim. In the range from 120 to 0.075%, the recovery was found to be quite constant and systematically heightened mainly due to the evaporation from vials during centrifuging and the displacement of solvent volume by the added matrix. Both system precision and repeatability remain almost constant in the interval from 120 to 10% at a R.S.D.% of 0.31 and 0.70%, respectively, indicating that the sample preparation is the major error source in this range (0.63%). Between 10 and 0.25%, a linear relationship between the logarithmized concentration and the repeatability was noted. However, for lower amounts close to the limit of quantitation, the R.S.D.% of measurements increases much more distinctly. This increase is caused by a strong rise of the system precision. At this concentration range, system precision and repeatability are not significantly different any longer. This leads to the conclusion that with the injection error being constant the peak integration error becomes the dominating error source at low concentrations, e.g. at concentrations below the five-fold of the LOQ. The results obtained here agree well with earlier published data. As the quantitation limit of 0.05% can be regarded as typical for a pharmaceutical impurity control test, generalizations of these findings from this extensive data set should be possible. In this context, peak integration and improvements of the signal-to-noise ratio are the most promising measures to improve an unsatisfactory precision in LC.

Keywords: Wide concentration range; Analytical uncertainty; Recovery; Precision; Error structure

1. Introduction

HPLC is probably the most important standard technique for quantitation in pharmaceutical analysis. Since the analytical uncertainty of all methods for quantitation has important consequences for the definition of acceptance criteria [1,2], there has always been considerable interest in the state-of-the-art LC precision. Numbers given for repeatability (RPT), which is the relative standard deviation (R.S.D.%) of measurements obtained from independently prepared samples within one series, typically range from 0.2 to 2% [3–8]. However, in certain cases a higher variability is found, e.g. due to matrix effects or low sample concentration. Then, it can be difficult to comply with standard acceptance criteria.

There is strong evidence that variability in LC is concentration-dependent. A linear relationship between the logarithmized concentration and the R.S.D.% has been suggested, however, on the basis of highly variable data from various sources [3–8]. Therefore, there has been plenty of discussion, if the suggestion could be generally valid.

* Corresponding author. Tel.: +49 531 391 2764; fax: +49 531 391 2799.
E-mail address: h.waetzig@tu-bs.de (H. Wätzig).

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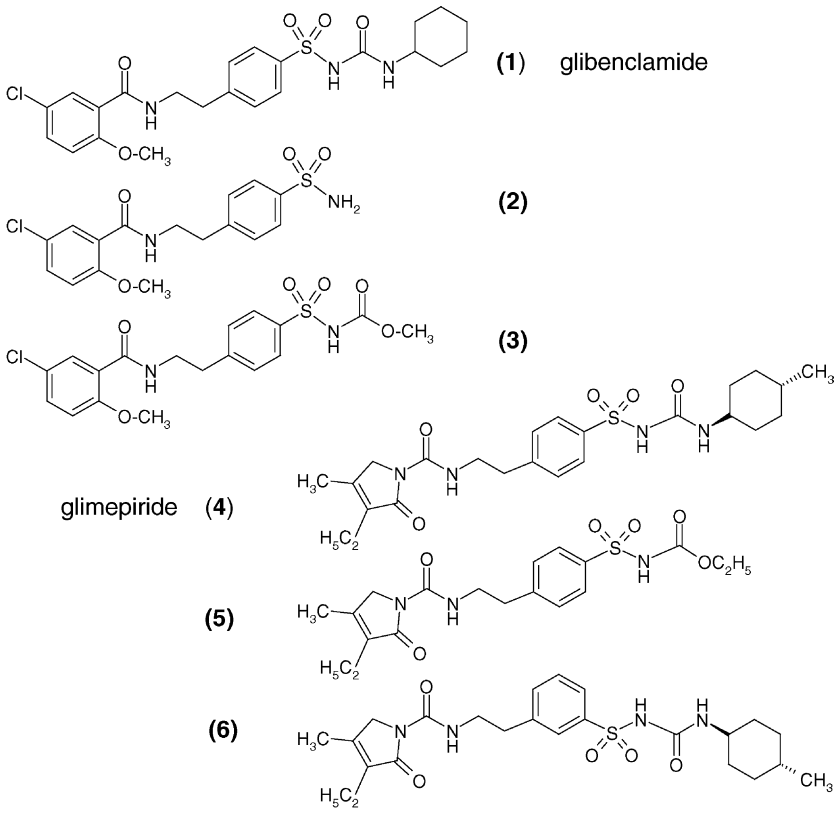

Fig. 1. Chemical structures of glibenclamide (**1**) and related substances **2**–**6**.

The sources for uncertainty in RP-HPLC for pharmaceutical product analysis have been comprehensively discussed [9]. In a recent study, upper limits for RPT and reproducibility have been determined to about 2 and 2.4% (R.S.D.%), respectively [10]. These numbers suggest that it is still important to better characterize the major error components in LC in order to improve precision. The control of the analytical uncertainty is crucial to comply with the 95–105% content standard acceptance criterion for European submissions of pharmaceutical formulations.

So far, no consistent study was carried out on LC precision over a wide concentration range. Although sample pretreatment and integration have often been discussed as major error sources, their quantitative contribution to the overall error was not obvious. What is more, it was not understood, from which concentration on the integration error becomes dominant. Therefore, a robust LC method was developed with a stable main compound and a set of well-characterized possible impurities to allow good generalizations of the results. Glibenclamide (**1**) (see Fig. 1) was found to be appropriate as the main compound.

The project consisted of three series (A, B and C) covering different concerns. In series A, recovery and precision were investigated for glibenclamide in the presence of a constant amount of typical tablet excipients in the context of a quantitation assay of a drug product. In series B, glibenclamide was analyzed with a more sensitive method. In series C, model impurities (**3**–**6**) were determined in the presence of glibenclamide simulating an impurity control test and to derive the influence of the different properties of the impurities on the course of precision.

2. Experimental

2.1. Chemicals

The following substances 1-[[4-[2-[(5-chloro-2-methoxybenzoyl)amino]ethyl]phenyl]sulfonyl]-3-cyclohexylurea (**1**) (purity >99.9%), 5-chloro-2-methoxy-*N*-[2-(4-sulfamoylphenyl)ethyl]benzamide (**2**) (99.0%), methyl[[4-[2-[(5-chloro-2-methoxybenzoyl)amino]ethyl]phenyl]sulfonyl] carbamate (**3**) (95.6%), 1-[[4-[2-[(3-ethyl-4-methyl-2-oxo-2,5-dihydro-1*H*-pyrrol-1-yl)carbonyl]-amino]ethyl]phenyl]sulfonyl]-3-(*trans*-4-methylcyclohexyl)urea (**4**) (99.7%), ethyl-[[4-[2-[(3 - ethyl-4-methyl-2-oxo- 2, 5-dihydro-1*H*-pyrrol-1-yl) carbonyl] amino] ethyl] phenyl] sulfonyl] carbamate (**5**) (99.5%) and 1-[[3-[2-[(3-ethyl-4-methyl-2-oxo-2,5-dihydro1*H*-pyrrol-1-yl)carbonyl]amino]ethyl]-phenyl]sulfonyl]-3-(*trans*-4-methylcyclohexyl)urea (**6**) (98.8%) (see Fig. 1) were provided by Aventis (Frankfurt, Germany). Acetonitrile for HPLC was purchased from Acros Organics (Gelnhausen, Germany) and from Fischer Scientific (Schwerte, Germany). Water for HPLC/LiChrosolv was

purchased from Merck (Darmstadt, Germany). Potassium dihydrogenphosphate, disodium hydrogenphosphate dihydrate, phosphoric acid 85% were purchased by Riedel-de-Häen (Seelze, Germany), lactose monohydrate, polyvidone 25000, microcrystalline cellulose and magnesium stearate by Merck, sodium starch glycolate and ferrum oxydatum flavum by Caelo (Hilden, Germany), respectively.

2.2. HPLC instrumentation

The HPLC system consisted of a solvent pump (model L 6200 A), an autosampler (AS 2000A), a diode array detector (L 7450) and an interface (D 6000 A) from Merck. The column oven (T1) was from Techlab (Erkerode, Germany). All separations were performed on a Superspher 100 RP-18 column (endcapped, 4 μm particle size, 125 mm × 4 mm, Merck). The data was collected and analyzed using the D 7000 HSM software (Merck).

2.3. HPLC method

2.3.1. Series A and C

The flow rate of the mobile phase and the column oven temperature were set at 1.25 mL/min and 35 °C, respectively. The injection volume was set to 10 μL. At first, the single injections of the typically six (n = 5 for one time; here one measurement was unsuccessful) standard solutions were carried out followed by a blank injection. For RPT, typically six (n = 5 for three times) independently prepared samples were injected once. After a second blank injection, six replicates of one sample for system precision (SYS) were carried out. The detection wavelength for **1**, **3** and **6** was 210 nm and for **4** and **5** it was 228 nm. Before each injection sequence, the column was rinsed with the mobile phase for 40 min at a flow rate of 0.5 mL/min and for 20 min at a flow rate of 1.25 mL/min. After each series, the column was rinsed for 30 min with (acetonitrile–water, 5:95, v/v) and afterwards for further 30 min with acetonitrile.

The lower concentration levels from 1.0 to 0.025% of series A were analyzed using the normal mode and also using the so-called low absorption mode of the L 7450 diode array detector, which provides a 10 times higher digital resolution of baseline and signals. The switching to the low absorption mode had no effect on the SYS.

2.3.2. Series B

For the series B, the above-mentioned method was performed, but instead of 10 μL an injection volume of 100 μL was used. Furthermore, two replicates of the samples have been made.

2.4. Sample preparation

2.4.1. Equipment

The weighing of substances in the interval from 0.375 to 12.5 mg was performed with a Sartorius microbalance (model 4431). For the upper weights (>31.25 mg), a Sartorius balance (BA210S) was used. The 1 mL dilution steps were performed by means of a pipette (model 1 mL, fix, Eppendorf). Furthermore, a centrifuge (5417C, Eppendorf) and an ultrasonic bath (model Bandelin Sonorex RK100) were used.

2.4.2. Mobile phase

The mobile phase (acetonitrile–buffer, 45:55, v/v) was prepared by dissolving 650 mg disodium hydrogen phosphate dihydrate in 550.0 g water and adding two drops of phosphoric acid 85% and 351.5 g acetonitrile to 1000 mL followed by degassing it in the ultrasonic bath. The resulting pH values of the 10 mM buffer before adding acetonitrile were of the interval 3.0–3.3. No influence between the pH value and the separation was observed.

2.4.3. Sample solvent

For the sample solvent (acetonitrile–buffer, 80:20, v/v), 200.0 g of 4 mM phosphate buffer (pH 7) and 625.0 g acetonitrile were dissolved to 1000 mL. The last dilution step during the series B (see Section 2.4.7) was performed with the sample solvent (acetonitrile–buffer, 40:60, v/v), which was of this composition: 600.0 g of 0.6 mM phosphate buffer (pH 7) and 312.4 g acetonitrile dissolved to 1000 mL.

2.4.4. Matrix

The matrix was used to simulate the quantitative analysis of an active ingredient in a drug product (tablet) in the series A and B. The matrix had the following composition: 74.6 mg of lactose monohydrate, 4.0 mg sodium starch glycolate, 0.5 mg polyvidone 25000, 10.0 mg microcrystalline cellulose, 0.5 mg magnesium stearate and 0.4 mg ferrum oxydatum flavum. The mixture was homogenized by pounding it in a mortar.

2.4.5. Standards

The standards and the corresponding samples were prepared at the same day. A standard of 100% was prepared by dissolving 5 mg to 25 mL with sample solvent. This 100% value corresponds to a sample solution of 0.2 mg/mL. A standard of 1% was prepared by dissolving 12.5 mg to 250 mL with sample solvent and subsequently diluting 1 mL of this solution to 25 mL. A standard of 0.1% was prepared by dissolving 12.5 mg to 100 mL with sample solvent and subsequently diluting 1 mL of this solution to 25 mL twice.

2.4.6. Samples of series A

The series A included the following concentration levels of glibenclamide: 120, 100, 80, 60, 40, 20, 10, 7.5, 5.0, 2.5, 1.0, 0.75, 0.50, 0.25, 0.10, 0.075, 0.050 and 0.025%. The preparation steps of these concentration levels are shown in Table 1. For all concentration levels, the 90 mg of the matrix were added before the last dilution step. Then, the sample suspensions were put for 15 min in an ultrasonic bath, centrifuged for 3 min at 14,000 rpm in open vials and transfilled for injection.

Table 1
Preparation steps of the series A and B

	Series A			Series B
Concentration level (%)	120–7.5	7.5–0.25	0.1–0.025	0.1–0.01
Initial weight (mg)	6.0–0.375	93.75–3.125	12.5–3.125	12.5–1.25
Stock solution (mL)	25	250	100	100
1. Dilution	None	1:25	1:25	1:25
2. Dilution	None	None	1:25	1:25[a]

[a] Sample solvent was acetonitrile–buffer (40:60, v/v).

2.4.7. Samples of series B

This series included the concentration levels 0.1, 0.05 and 0.01%. The preparation steps for this series are also shown in Table 1. For the last dilution step, the sample solvent (acetonitrile–buffer, 40:60, v/v) was used.

2.4.8. Samples of series C

Every sample in the series C consisted of an independently prepared complex mixture of various related substances and glibenclamide as the matrix for simulating the quantitative analysis of impurities in a drug substance. The concentration levels covered by this series were 1.0, 0.75, 0.50, 0.25, 0.10, 0.075, 0.050 and 0.025%. The involved compounds were **3**–**6**. To assure an initial weight of more than 1 mg, the preparation steps have been changed compared to series A. The corresponding preparation steps for the different levels are shown in Table 2. Additionally, for simulation of drug substance, 100% glibenclamide was added as a matrix before each last dilution step.

2.5. Data evaluation

For data evaluation, the percentage recovery of a single measurement arises as the ratio of found to added substance multiplied by 100. The amount of the found substance results as the ratio of the specific area of a single measurement to the mean of the specific areas of the standards (so-called single-point calibration). In series A, for the sample concentration levels from 120 to 7.5%, a standard concentration of 100% was taken. In the next range from 7.5 to 0.025% as well as in series C, the standard concentration was 1%. For series B, a standard of 0.1% was used. Response factors for the impurities were determined as means of peak area ratios calculated from 0.5 to 1% solutions on three different days. Here, values of 0.97 for **3**, 1.72 for **4**, 1.37 for **5** and 1.85 for **6** were obtained.

Table 2
Preparation steps in series C

	Series C		
Concentration level (%)	1.0–0.25	0.1–0.05	0.025
Initial weight (mg)	5.0–1.25	3.125–1.563	3.125
Stock solution (mL)	100	25	100
1. Dilution	1:25	1:25	1:25
2. Dilution	None	1:25	1:25

RPT was calculated as the relative standard deviation of peak area of the six sample preparations and the SYS as the relative standard deviation of the six replicate injections of one sample solution. Integration by means of the D 7000 HSM software system was not suitable for the lowest investigated concentrations. In order to obtain comparable results, manual integration was carried out throughout this work. For statistical calculations and the compilation of all data, Microsoft Excel was used.

3. Results and discussion

3.1. HPLC method development

The HPLC method used throughout this work (see Section 2.3) was developed to achieve an efficient, robust and reliable separation of the substances used (Fig. 2). In order to increase the sample stability, a phosphate buffer (pH 7) was added to the sample solvent (see Section 2.4.3). The speed of degradation of glibenclamide to **2** was reduced from

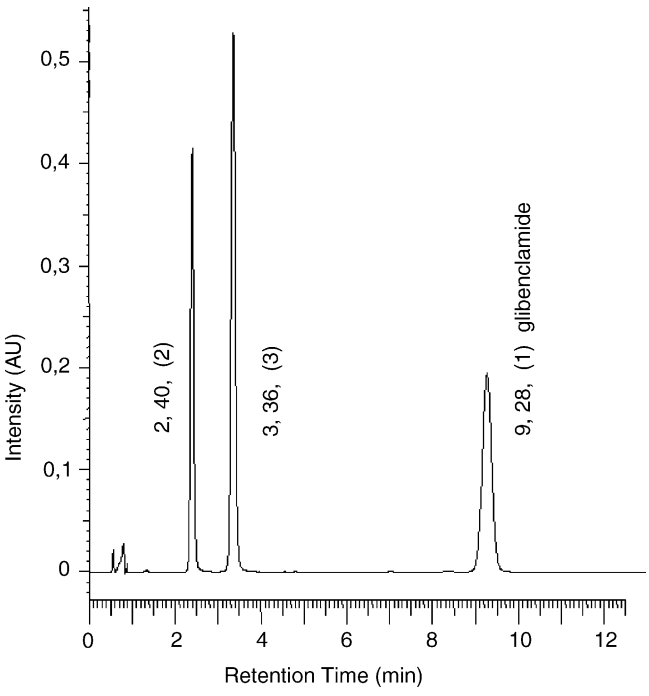

Fig. 2. Chromatogram of glibenclamide (**1**) next to **2** and **3**.

about 0.05 to below 0.01%/h. A pH of 7 showed the best reducing effect (within 3 days) of the investigated pH values 3, 5 and 7. Moreover, a column oven was integrated into the HPLC system. Using this oven, the SYS improved from 0.49 to 0.31%.

3.2. Relationship between sample concentration and precision

3.2.1. Series A: concentration levels from 120 to 0.025%

Solutions of glibenclamide starting from 120% label claim (see Section 2.4.6) were prepared, stepwise lowering the concentrations until the condition S/N > 2 could just be met. The R.S.D.% values for RPT and SYS (see Section 2.3) over the concentration range are shown in Fig. 3. The concentration range can be divided in three areas. First, there is the range from 120 to 10%, where SYS and RPT remain remarkably constant (see small diagram in Fig. 3). In the range from 7.5 to 0.25%, one can see a clear increase of the RPT (an increase of RPT, given as R.S.D.%, means worsening), similar to the linear relationship between the logarithmized concentration and the precision described by HORWITZ [5–8]. Below 0.25%, the RPT increases very strongly (Fig. 3). Switching the digital resolution (normal to low mode, see Section 2.3.1) which was necessary to cover the whole concentration range, showed no effect on the SYS.

In the range from 7.5% to the lowest investigated concentration level of 0.025%, the numbers for SYS and RPT do not significantly differ due to their large confidence intervals. For $n - 1 = 5$ degrees of freedom ($n = 6$ measurements) each and an error probability α of 0.05, the ratio of the R.S.D.% must exceed 1.85 to show a significant difference. Considering SYS and RPT at $c = 0.5\%$, for example, and applying the F-test with the values 1.89% for RPT and 1.46% for SYS, this ratio is just 1.29.

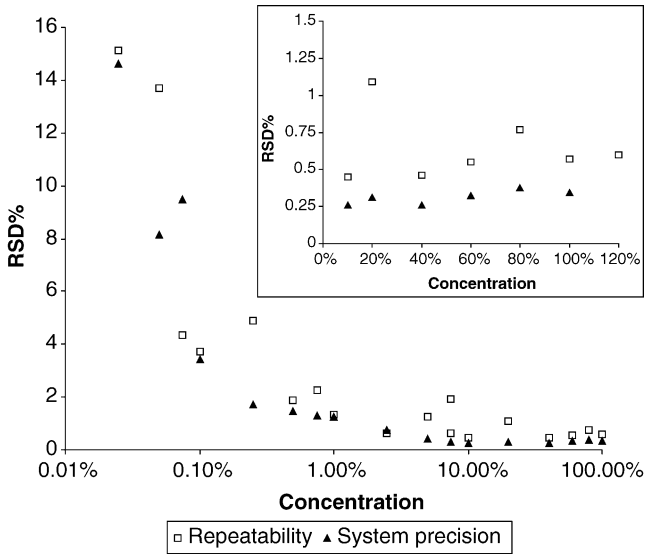

Fig. 3. Repeatability and system precision over the whole range. For 7.5%, there are two values for RPT, one for the range 120–7.5%, the other for 7.5–0.025%; between these ranges, the sample preparation changed (see Table 1). The magnification in the upper right corner shows the repeatability and system precision from 120 to 10%.

Table 3
R.S.D.% values for system precision of **1**, **3**, **4** and **6**

	1.0%	0.75%	0.50%	0.25%	0.10%	0.075%
1	1.24	1.30	1.46	1.70	3.42	9.47
3	0.45	0.80	0.79	1.33	1.85	3.46
4	1.18	2.47	2.46	3.31	7.66	12.86
6	0.60	1.66	2.12	4.37	6.15	9.98

Comparing SYS and RPT in pairs in the range from 10 to 100%, the difference is not always significant as well. However, this is mainly due to the low number of the degrees of freedom. After pooling all SYSs and RPTs in that range (see Section 3.3), the remaining difference becomes significant as can be seen by the calculated value of 2.26, which is accordingly much larger than the limit 1.36 (degrees of freedom = 30, $\alpha = 0.05$). Therefore, the sample preparation is a significant error source in this range and, as can be shown in Section 3.3, even the major one.

The limit of quantitation (LOQ; lowest concentration that can be quantified with a R.S.D.% < 10%) was determined as 0.05%. The signal-to-noise ratio was 6 at this level [11].

3.2.2. Series B: concentration levels from 0.1 to 0.01%, method with enhanced sensitivity

The increase of sensitivity by the factor of 10 was accomplished by changing the injection volume from 10 to 100 μL. An initially occurring peak broadening was compensated by reducing the acetonitrile concentration within the sample solution (see Section 2.4.7). A clear improvement of RPT in series B was observed. The R.S.D.% values of series B are of the same order of magnitude as those of series A corresponding to 10 times higher concentration levels (e.g. the R.S.D.% values of 1% in series A was about the same as the value of 0.1% in series B).

3.2.3. Series C: related substances next to glibenclamide as matrix from 1 to 0.025%

A typical chromatogram containing the substances **3**–**6** next to main compound **1** is shown in Fig. 4. In principle, the related substances show the same behavior as glibenclamide concerning the relationship of RPT, SYS and concentration in the investigated range. However, there are certain differences between these substances concerning the level of R.S.D.% from which the strong increase starts (Fig. 5). The different behavior of this increase in RPT can be explained by means of the parameters UV absorbance (compare **1** and **6** in Table 3 and Fig. 6), retention time (compare **1** and **3** in Table 3 and Fig. 6) and detection wavelength and related noise (compare **4** and **6** in Table 3, detected at 228 and 210 nm, respectively). Here, the retention times and the UV absorbances are close to each other. The observed difference in SYS is probably due to the almost two-fold noise at 228 nm. The model impurity

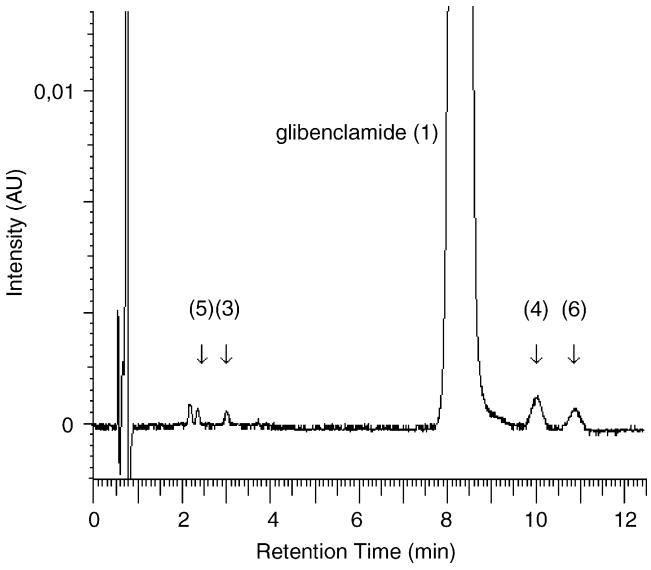

Fig. 4. Chromatogram of glibenclamide (**1**) and related substances **3**–**6** (series C) in the concentration levels: **1** = 100%; **3** = 0.025%; **4** = 0.500%; **5** = 0.075%; **6** = 0.250%.

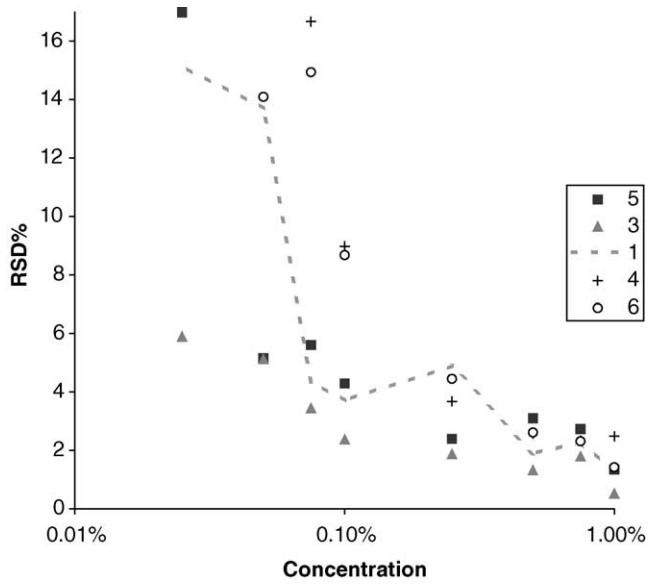

Fig. 5. RPT values from **1** (dashed graph) and **3**–**6**.

5 was purposefully chosen as it is just baseline-separated from the degradation product **2**. This is possibly a reason for the higher R.S.D.% values of **5** compared to **3**. However, the other parameters are different here as well. Therefore, an additional effect on the SYS due to the neighboring peak cannot be assured.

3.3. Error components

The main components of the RPT as the total within-day variation (Sections 2.5 and 2.3.1) consist of sample preparation, injection and integration error. All values for RPT and SYS, reported as R.S.D.%, are shown in Fig. 3. In the concentration range from 100 to 10% RPT and SYS remain constant, pooled values of 0.70 and 0.31% were found, respectively. According to the Gauss's law of error propagation, the total variance is the sum of the variance components:

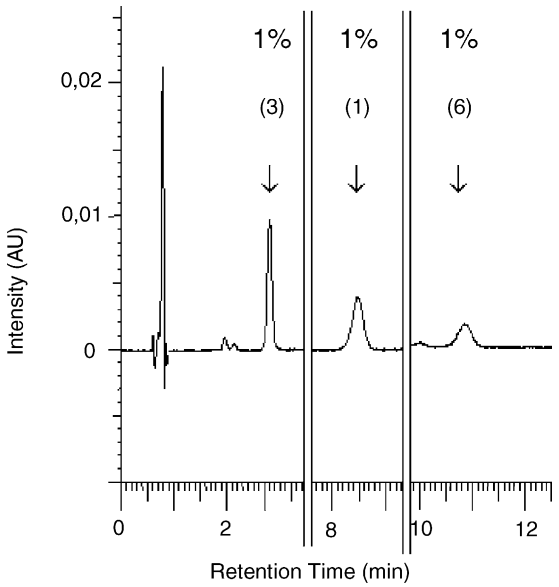

Fig. 6. Overlay of the peaks **1**, **3** and **6** from different runs in a concentration of 1%.

$$\hat{\sigma}^2_{\text{ges}} = \sum \hat{\sigma}^2_i \quad (1)$$

Here, the total variance corresponds to 0.50 (squared RPT). Error components other than sample preparation are included in SYS, the corresponding variance being 0.10. The difference in variance, 0.40, is thus due to sample preparation. This value corresponds to an R.S.D.% of about 0.63% ($\sqrt{}$0.40). Therefore, the sample preparation can be considered as the main source of error in this concentration range. The contribution of the sample preparation for itself is considered to be nearly constant, since for the three concentration ranges (see the three columns in series A in Table 1) only the initial weights have been changed and the respective preparations only differ in a further dilution step. Further, SYS remains almost constant in the range from 100 to 10%, though the sample concentration and thus the peak area decreases to one-tenth (Fig. 3). Therefore, the contribution of the integration error to the total variance must be minor in this range, otherwise one would have seen an increase of SYS. Hence, the only relevant remaining error source included in SYS is the injection error of this system. The obtained value of 0.31% for SYS is therefore a good estimate for the contribution of the injection error for all investigated scenarios. For lower concentrations ($\leq$0.5%), the total error increases dramatically. Therefore, the contribution of the sample preparation to the total error becomes very small. According to Eq. (1), considering a typical total variation of more than 2% for this range, a value of 0.63% for sample preparation contributes less than 10% (Fig. 7), corresponding to a concentration level below 0.5%. The injection error being constant, the integration er-

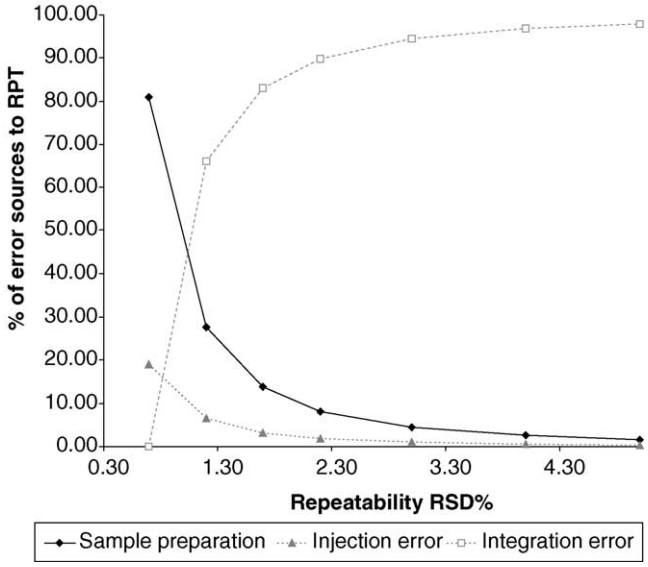

Fig. 7. Error contributions to increasing repeatability. A constant sample preparation error and injection error of 0.63 and 0.31% (R.S.D.%) are assumed.

ror becomes the dominating error source in this concentration range. This has been confirmed by series B. By increasing the injection volume and thus the peak area by a factor of 10 and keeping all other experimental parameters constant, the error curve was shifted by this factor of 10 (see Section 3.2.2). Moreover, the SYS of **1** from series A has been evaluated at the different wavelengths 210, 228 and 240 nm. The lower UV absorbances of 228 and 240 nm compared to 210 nm can be seen in Fig. 8. The noise was found to be wavelength-independent. The consequences for each SYS is depicted in Fig. 8 as well. Using the more unfavorable wavelength, the

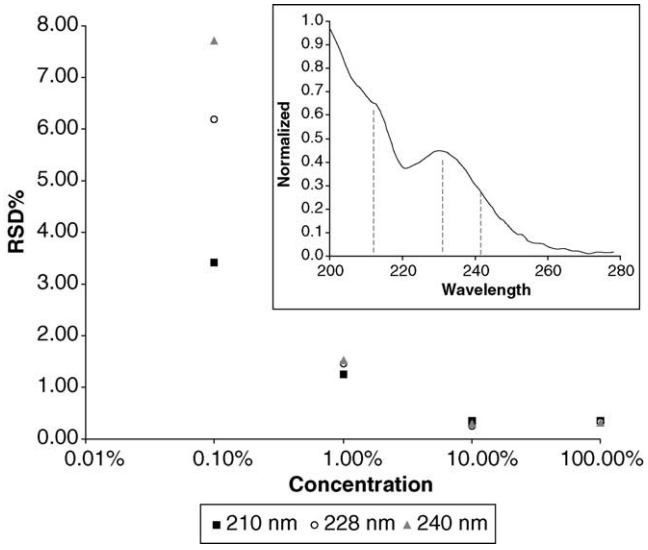

Fig. 8. System precision of **1** at different wavelengths. The small figure shows the UV spectrum of **1**, taken from DAD data obtained during HPLC experiments (see Section 2.2). Dashed lines: evaluation wavelengths 210, 228 and 240 nm.

peak area and thus the signal-to-noise ratio decreases. This leads to an increase of the integration error, becoming manifest in a higher SYS. The ratios of the UV absorbances are 2.2:1.4:1 for 210, 228 and 240 nm, respectively. The signal-to-noise ratio at 210 nm is about twice the value at 240 nm for the same concentration. Comparing an evaluation at 240 nm to another at 210 nm and using half the concentration, about the same signal-to-noise ratio is obtained. At lower concentrations, the SYS is only dependent on the signal-to-noise ratio, independently what concentrations and wavelengths are considered. Here, proper integration is especially important to achieve satisfactory analytical results.

3.4. Relation between sample concentration and recovery

The recovery was found to be systematically heightened about 1.5% over the whole investigated interval. This systematic effect, however, occurred to be quite constant, considering the observed increase in variability at lower concentration. The changing of the standard concentration (first 100%, then 1%) for contents below 7.5% did not have any effect.

In order to investigate the reason for this biased recovery, all matrix components were separately investigated. Samples consisted of glibenclamide and the respective matrix component in the same amount like in the complete matrix (see Section 2.4.4). Surprisingly, each matrix component as such led to the same above-mentioned heightened recovery, even 0.4 mg of $Fe_2O_3 \cdot H_2O$. Thus, none of the chemicals could be the reason but a step in the sample work-up which was different for standards and samples with matrix. The earlier not as error source considered centrifugation of the matrix-containing sample (Section 2.4.6) was found as the reason for the elevated recovery. Obviously centrifugation leads to solvent evaporation and concentration of samples. This also explains why the same recovery was found at all different concentration levels.

In order to confirm this, another set of four standard solutions and four samples at the 100% level was prepared (Section 2.4.6). In this experiment, however, all solutions were centrifuged. The differences in areas between standards and samples became negligible, when just solutions from open or closed vials were compared. A recovery of 100.5% was derived from this series. Hence, this reason for the biased recovery was clearly identified.

A perceptible increase of the recoveries of related substances **3**–**6** for lower concentrations can be assumed, possibly due to effects from integration difficulties and biased baseline setting. Note that baseline shifts can also cause deviations from linearity [12].

4. Conclusions

The investigation of precision and recovery over such a wide concentration range provides considerably new insights

into the error structure, which changes at different concentration levels. In the first concentration range of series A, above 10% of the typical concentration for this assay, the RPT and the SYS remain constant at 0.70 and 0.31%, respectively. The integration error is of no importance. Therefore, the value of 0.31% for SYS can be assigned to the injection error. The sample preparation, determined to 0.63%, dominates the RPT. Similar results for higher sample concentrations were obtained in [9].

All additional variation below 10% arises from the integration error, which can be assumed as the only variable error source. During the second range of 7.5–0.25%, a continuous increase of the RPT as well as the SYS could be noted, as described by the HORWITZ relationship. During this range, the dominating error source changes from sample preparation to integration. In the concentration range below 0.25%, the integration becomes the clearly dominating error source. At such concentration levels, the increase in sensitivity is the only way to improve the RPT (shown in series B). In series C, the related substances show the same principal behavior concerning precision and recovery.

The recovery remains systematically heightened and constant down to a concentration level of 0.075%. Below this level, distinctly deviations of recovery from 100% for series A and C occurred due to the difficulties of unbiased baseline setting at the limit of quantitation.

This study demonstrates, that sample preparation and integration are the major error sources in LC (see Fig. 7), becoming dominant at high and low concentrations, respectively. If relatively high percentage R.S.D.% values (e.g. 2%) shall be improved at concentrations below the five-fold of LOQ, it is most promising to consider integration as the dominating factor. This is confirmed, if SYS and RPT are of about the same size. Measures such as improving detection and integration, as well as sample pre-concentration can then be taken to improve LC precision.

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