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LIMITS OF THE INTERNAL STANDARD TECHNIQUE IN CHROMATOGRAPHY

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SUMMARY

By use of the law of propagation of error it is possible to determine how the internal standard technique affects the reproducibility of a given chromatographic analysis. The usefulness of this procedure is demonstrated with practical examples. Guidelines are provided for the proper application of the internal standard technique in the analysis of drugs in biological materials. It can be shown that, in practice, an external calibration is often advantageous compared to the internal standard technique.

INTRODUCTION

The internal standard technique is widely used in chromatography. In this approach an accurate amount of a known compound is added to the sample solution prior to analysis. Thereby errors in the analytical measurement are often reduced, since any loss of sample is compensated by the loss of an equivalent amount of internal standard. Instead of the absolute value of the peak height (or peak area), the ratio of the peak height of the compound to the peak height of the internal standard is used in calibration and in the evaluation of the unknown samples.

In gas chromatography (GC), where small quantities (often only a few microlitres) are injected onto the column, the internal standard technique considerably improves the reproducibility of determinations. The volumes injected in high-performance liquid chromatography (HPLC) are much higher and yield a better precision of injection. With modern sample injectors, the precision of injection is reported to be 0.3% for partial loop filling and 0.05% for complete loop filling[1]. These excellent results were obtained without the use of an internal standard. Therefore the question arises as to whether, in HPLC procedures, the addition of an internal standard is necessary.

Furthermore, some critical remarks have recently been published[2] about the usefulness of the internal standard technique in the analysis of drugs. In the present paper it will be shown by means of the law of propagation of error how an internal standard can improve or impair the reproducibility of an assay. The application of the formulae is illustrated by experimental data.

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THEORETICAL

Law of propagation of error in the internal standard technique

Some simple formulae may be used to get information about the precision of the quantitative evaluation of chromatographic procedures, both with and without an internal standard.

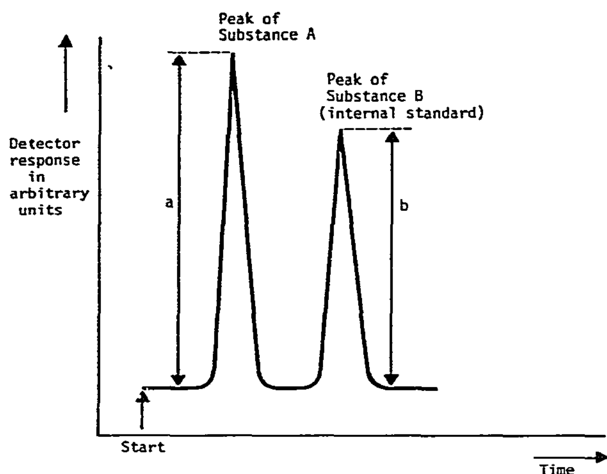

Fig. 1. Chromatogram of a substance A and internal standard B.

In Fig. 1 a chromatogram is shown of two substances. This record could originate from a GC, HPLC or thin-layer chromatographic separation. Peak A is the signal of the substance to be determined and peak B that of the internal standard. The corresponding peak heights (or peak areas) are a and b. If the same sample amount is chromatographed repeatedly, the peak heights show distinct variations. A measure of this variation, corresponding to the precision of the determinations, is the relative standard deviation, also called the coefficient of variation. The following symbols and definitions will be used.

For peak A (substance to be determined) the standard deviation of the peak height a

$$s_a = \sqrt{\frac{\sum_i^n (a_i - \bar{a})^2}{n - 1}} \tag{1}$$

where n is the number of the individual measurements a_i. The relative standard deviation of a

$$s_{a,\mathrm{rel}} = s_a/\bar{a} \tag{2}$$

where $\bar{a} = \sum_i^n a_i/n$, the mean of individual values.

For peak B (internal standard) the standard deviation of the peak b

$$s_b = \sqrt{\frac{\sum_i^n (b_i - \bar{b})^2}{n - 1}} \qquad (3)$$

where n is the number of the individual measurements b_i. The relative standard deviation of b

$$s_{b,\mathrm{rel}} = s_b/\bar{b} \qquad (4)$$

where $\bar{b} = \sum_i^n b_i/n$, the mean of the individual values.

In the internal standard technique the quotient Q_i, rather than the absolute values a_i, is used:

$$Q_i = a_i/b_i$$

For this quotient the standard deviation

$$s_Q = \sqrt{\frac{\sum_i^n (Q_i - \bar{Q})^2}{n - 1}} \qquad (5)$$

where n is the number of the individual ratios Q_i, and the relative standard deviation

$$s_{Q,\mathrm{rel}} = s_Q/\bar{Q} \qquad (6)$$

where $\bar{Q} = \sum_i^n Q_i/n$. If the internal standard technique improves the precision of a method

$$s_{Q,\mathrm{rel}} < s_{a,\mathrm{rel}} \qquad (7)$$

or:

$$s^2_{Q,\mathrm{rel}} < s^2_{a,\mathrm{rel}} \qquad (7')$$

It is obvious that the relative standard deviation of the quotient, $s_{Q,\mathrm{rel}}$, is related to the relative standard deviations $s_{a,\mathrm{rel}}$ and $s_{b,\mathrm{rel}}$. From the theory of propagation of error[3,4]

$$s_Q^2 \approx \left(\frac{\bar{a}}{\bar{b}}\right)^2 \left[\left(\frac{s_a}{\bar{a}}\right)^2 + \left(\frac{s_b}{\bar{b}}\right)^2 - 2 \cdot \frac{s_{ab}}{\bar{a}\bar{b}}\right] \qquad (8)$$

where $s_{ab} = (1/n - 1) \sum_i^n (a_i - \bar{a})(b_i - \bar{b})$. When $\bar{a}/\bar{b}$ is approximated by $\bar{Q}$, relation 8 can be transformed to:

$$s^2_{Q,\mathrm{rel}} \approx s^2_{a,\mathrm{rel}} + s^2_{b,\mathrm{rel}} - 2 \cdot \frac{s_{ab}}{s_a s_b} \cdot \frac{s_a}{\bar{a}} \cdot \frac{s_b}{\bar{b}} \tag{9}$$

Using the definition[4,5] of the correlation coefficient $r = s_{ab}/s_a s_b$ one obtains:

$$s^2_{Q,\mathrm{rel}} \approx s^2_{a,\mathrm{rel}} + s^2_{b,\mathrm{rel}} - 2rs_{a,\mathrm{rel}}s_{b,\mathrm{rel}} \tag{10}$$

This approximation approaches equality as $s_{a,\mathrm{rel}}$ and $s_{b,\mathrm{rel}}$ tend to zero. In general, values of less than 10% are allowable. The approximation for $s^2_{Q,\mathrm{rel}}$ can be substituted in eqns. 7 and 7′ respectively

$$s^2_{a,\mathrm{rel}} + s^2_{b,\mathrm{rel}} - 2rs_{a,\mathrm{rel}}s_{b,\mathrm{rel}} < s^2_{a,\mathrm{rel}}$$

and rearrangement yields:

$$s_{b,\mathrm{rel}} < 2rs_{a,\mathrm{rel}} \tag{11}$$

Only if this relation holds true will an internal standard procedure improve a particular method.

The correlation coefficient is within the limits[5] $-1 \leqslant r \leqslant +1$. Furthermore $s_{a,\mathrm{rel}}$ and $s_{b,\mathrm{rel}}$ are positive, therefore the relation 10 can only holds true if $r > 0$. This requirement is obvious, since a positive correlation must exist between the peak height of the internal standard and of the analyte.

Some special cases are now discussed.

(1) *Correlation coefficient $r = +1$.* There is a strong linear correlation between the peak heights a and b. Formula 11 yields $s_{b,\mathrm{rel}} < 2s_{a,\mathrm{rel}}$. This means that the precision of a method cannot be improved with an internal standard, even under best correlation, if the relative standard deviation of the internal standard is more than twice the relative standard deviation of the substance to be determined.

(2) *The relative standard deviations of the substance to be determined and of the internal standard are of equal size: $s_{a,rel} = s_{b,rel}$.* In this case formula 11 gives $r > 0.5$. Only if the correlation coefficient between a and b is larger than 0.5 will use of the internal standard improve the precision.

(3) *The relative standard deviation of the substance to be determined is twice that of the internal standard.* In this case, use of the internal standard improves the precision of the method, if $r > 0.25$. This finding can be generalized: The smaller $s_{b,\mathrm{rel}}$ is compared to $s_{a,\mathrm{rel}}$, the weaker will be the correlation between a and b.

Practical examples will illustrate the usefulness of formula 11. In all the cases described below, the relative standard deviations are expressed as a percentage.

Practical examples

In many cases the relative standard deviations s_a and s_b are not calculated and only $s_{Q,\mathrm{rel}}$ is evaluated, but the data required to calculate $s_{a,\mathrm{rel}}$ and $s_{b,\mathrm{rel}}$ are available. With the small advanced calculators of today it is possible to get all the parameters needed with minimal effort.

Example 1: HPLC with manual injection and partial loop filling. The same volume (100 μl) of a mixture of the substance to be determined and the internal standard was injected several times and chromatographed. The loop volume was 200

TABLE I

EXAMPLE 1: HPLC WITH MANUAL INJECTION AND PARTIAL LOOP FILLING

Correlation coefficient, $r = +0.949$.

Injection No.	*Peak height, a, of substance A (mm)*	*Peak height, b, of substance B (internal standard) (mm)*	*Peak height ratio, Q*
1	96	98	0.9796
2	101.5	104	0.9760
3	100	103	0.9702
4	100.5	104	0.9663
5	98.5	102	0.9657
6	101	104	0.9712
7	100	102	0.9804
8	97.5	101	0.9653
9	98.5	101.5	0.9704
10	101.5	104.5	0.9713
11	100	104	0.9615
12	101	104.5	0.9665
	$\bar{a} = 99.7$	$\bar{b} = 102.7$	$\bar{Q} = 0.9704$
	$s_{a,\text{rel}} = 1.7\%$	$s_{b,\text{rel}} = 1.9\%$	$s_{Q,\text{rel}} = 0.60\%$

μl. The calculated peak heights are in Table I. To interpret the results the individual relative standard deviations of signals A and B and the correlation coefficient have to be calculated. They are: $s_{a,\text{rel}} = 1.7\%$, $s_{b,\text{rel}} = 1.9\%$ and $r = +0.949$. Substitution of these values in formula 9 gives an estimation of the relative standard deviation of the ratio: $s^2_{Q,\text{rel}}$ (approx.) $\approx 1.7^2 + 1.9^2 - 2 \cdot 0.949 \cdot 1.7 \cdot 1.9$, *i.e.*, $s_{Q,\text{rel}}$ (approx.) $= 0.61\%$. This value is in good agreement with the value (0.60%) found directly with formula 6.

In this example the precision of the volume of injection is the limiting factor and the internal standard technique improves the reproducibility, since the relative standard deviation of the substance to be determined ($s_{a,\text{rel}} = 1.7\%$) is much larger than $s_{Q,\text{rel}}$ (0.60%). This shows that the imprecision due to the variation of the injection volume can largely be eliminated by use of an internal standard. There exists a high correlation between the peak heights a and b ($r = 0.949$), $s_{a,\text{rel}}$ and $s_{b,\text{rel}}$ are of the same order of magnitude and therefore the internal standard is appropriate (special cases 1 and 2 respectively).

Example 2: as in example 1, but automatic injection with complete loop filling (100 μl). The peak height measurement was done by a data system. The data are summarized in Table II. The following values were calculated: $s_{a,\text{rel}} = 0.82\%$, $s_{b,\text{rel}} = 0.89\%$ and $r = +0.496$. Formula 9 yields $s_{Q,\text{rel}}$ (approx.) $\approx 0.87\%$. This is again in good agreement with the value (0.81%) calculated directly from formula 6.

In contrast to example 1, use of the internal standard does not improve the precision of the method. The reason is that, when $s_{a,\text{rel}}$ and $s_{b,\text{rel}}$ are of equal magnitude, then the correlation coefficient must be larger than 0.5 for the internal standard to improve the precision of a procedure (special case 2). This example shows that when an automatic sample injector is used in HPLC the precision of the injection

TABLE II

EXAMPLE 2: HPLC, AUTOMATIC INJECTION WITH COMPLETE LOOP FILLING

Correlation coefficient, $r = +0.496$.

Injection No.	*Peak height, a, of substance A (arbitrary units)*	*Peak height, b, of substance B (internal standard) (arbitrary units)*	*Peak height ratio, Q*
1	1696	1771	0.9577
2	1685	1774	0.9498
3	1665	1772	0.9396
4	1648	1737	0.9488
5	1658	1754	0.9453
6	1678	1746	0.9611
7	1672	1727	0.9682
8	1663	1744	0.9536
9	1673	1756	0.9527
10	1677	1749	0.9588
	$\bar{a} = 1672$	$\bar{b} = 1753$	$\bar{Q} = 0.9536$
	$s_{a,rel} = 0.82\%$	$s_{b,rel} = 0.89\%$	$s_{Q,rel} = 0.87\%$

volume is not the limiting factor for the reproducibility and the internal standard brings no evident advantages.

Example 3: HPLC of plasma extracts. Aliquots of plasma, which had been spiked with the substance to be determined, were mixed with the internal standard and extracted. The extracts were injected with an automatic sample injector with

TABLE III

EXAMPLE 3: HPLC OF PLASMA EXTRACTS WITH AUTOMATIC INJECTION AND COMPLETE LOOP FILLING

Correlation coefficient, $r = -0.672$.

Injection No.	*Peak height, a, of substance A (mm)*	*Peak height, b, of substance B (internal standard) (mm)*	*Peak height ratio, Q*
1	114	119	0.9580
2	112	120	0.9333
3	112	120	0.9333
4	112	120	0.9333
5	112	121	0.9256
6	112	123	0.9106
7	110	122	0.9016
8	111	124	0.8952
9	111	124	0.8952
10	111	125	0.8880
11	111	126	0.8801
	$\bar{a} = 111.6$	$\bar{b} = 122.2$	$\bar{Q} = 0.9140$
	$s_{a,rel} = 0.92\%$	$s_{b,rel} = 1.9\%$	$s_{Q,rel} = 2.6\%$

complete loop filling (100 μl). The peak heights found are summarized in Table III. The following data were calculated: $s_{a,rel}$ = 0.92%, $s_{b,rel}$ 1.9% and r = −0.672. Formula 9 gives $s_{Q,rel}$ (approx.) ≈ 2.6%, which is equal to the value (2.6%) found with formula 6.

In this example, the internal standard procedure considerably impairs the precision of the assay; instead of $s_{a,rel}$ = 0.92% a precision of $s_{a,rel}$ = 2.6% is obtained There are two reasons for this phenomenon. On the one hand $s_{b,rel}$ is more than twice $s_{a,rel}$; as mentioned under special case 1, even with the best correlation (r = 1) use of the internal standard cannot improve the precision under these circumstances. On the other hand, there r is negative and therefore there is no correlation between the signals of substances A and B in the chromatogram. The reasons for this finding will be discussed below.

Example 4: GC of plasma extracts. The following example, from the GC determination of a drug, illustrates the general applicability of formula 11. Plasma

TABLE IV

EXAMPLE 4: GC OF PLASMA EXTRACTS WITH AUTOMATIC INJECTION

Correlation coefficient, r = +0.558.

Injection No.	*Peak area, a, of substance A (arbitrary units)*	*Peak area, b, of substance B (internal standard) (arbitrary units)*	*Peak area ratio, Q*
1	2109	1338	1.576
2	2056	1356	1.516
3	1970	1272	1.549
4	1937	1285	1.507
5	1757	1285	1.367
6	1984	1315	1.509
7	1943	1294	1.502
8	1884	1332	1.414
9	1942	1308	1.485
	$\bar{a}$ = 1954	$\bar{b}$ = 1309	$\bar{Q}$ = 1.492
	$s_{a,rel}$ = 5.1%	$s_{b,rel}$ = 2.2%	$s_{Q,rel}$ = 4.3%

extracts containing an internal standard were injected with an automatic sample injector and the peak areas measured with a data system. The results are compiled in Table IV. The values calculated were: $s_{a,rel}$ = 5.1%, $s_{b,rel}$ = 2.2% and r = +0.558. From formula 9, $s_{Q,rel}$ ≈ 4.3%, the same value as calculated directly from the ratios of Table IV.

The relative standard deviation of the internal standard, $s_{b,rel}$, is considerably smaller than $s_{a,rel}$ and therefore a correlation coefficient of +0.556 is sufficient to improve the precision of the procedure with an internal standard.

DISCUSSION

The variations of the injection volume can be overcome by the internal standard technique as demonstrated by example 1. But in many chromatographic assays

the injection volume is not the only source of the methodological variations. The chromatographic systems are often responsible for fluctuations which are independent of the injection volume. In example 3 the relative standard deviation of the internal standard is larger than that of the substance being analysed. The chromatographic conditions, which are not optimal for the internal standard, are one reason for this effect. The peak shape deviates in some cases from the ideal gaussian curve. Possibly, peak area measurement could improve the precision in this case. However, the main point in this example is the variation of the extraction. Even though the internal standard and the substance to be determined are similar compounds, the extraction behaviour is different and is responsible for the higher relative standard deviation. This phenomenon can be often observed in the analysis of drugs in biological materials[2]. The extraction conditions and the chromatographic system could be changed in example 3. However, this is no guarantee that the precision will be improved, since all the conditions described below must be met. In this example, therefore, external standardization provides the best precision. In the analysis of drugs and metabolites in biological materials the precision of the assays is often in the range of 2–5%. In example 3 a relative standard deviation of 0.9% was found for substance A. It is unlikely that the precision would be improved using an internal standard.

Curry and Whelpton[2] have mentioned several important points which are often overlooked. It is often fallacious to use a second compound as internal standard for checking the extraction, stability or derivatization: "it is naive to expect two compounds (even homologous) to exhibit identical chemistry —be it extraction, derivatization or stability— and there is no reason to suppose the inclusion of an internal standard will inevitably produce a more satisfactory assay. The probability that the internal standard adversely affects the data should even be considered". Example 3 demonstrates that these remarks are well-founded.

Guidelines for application of the internal standard technique in the assay of drugs in biological materials

The following are general requirements of the internal standard:

(i) It must be completely resolved in the chromatogram from the other known and unknown substances

(ii) It must be eluted near the peak of interest

(iii) The peak height (or peak area) of the internal standard must be similar to that of the substance to be determined

(iv) It must be chemically similar to the substance of interest

(v) It must be chemically stable

The following points have to be observed in the analysis of drugs in biological materials:

(i) The internal standard must be added to the biological samples in solution. Whenever possible, aqueous solutions should be used. After addition, the samples must be mixed thoroughly to obtain a uniform distribution of the internal standard

(ii) If there are extraction steps prior to chromatography, the internal standard should show similar behaviour to the analyte; namely, the partition coefficients should be equal

(iii) The internal standard should not be a metabolite of the drug of interest

(iv) The internal standard should not interfere with metabolites of the drug or endogenous compounds

Unsuitable approaches are as follows:

(i) Evaporation of an organic solution of the internal standard and addition of the biological sample to the residue followed by mixing is not recommended. It is impossible to guarantee in each case a complete dissolution of the internal standard under these circumstances.

(iii) The addition of an "internal standard" to the extract after extraction prior to chromatography is only of limited value in HPLC, since with modern injectors excellent precision of the injection volume is obtained. The variation of the injection volume is small, compared to the variation of the clean-up steps. An exception is the case where the extract is injected in a volatile solvent. The addition of an "internal standard" can eliminate evaporation losses.

CONCLUSIONS

The aim of this study was to show that the internal standard technique will not inevitably improve the precision of an assay. In every case the relative standard deviation of both the analyte and internal standard should be considered as well as $s_{a,\mathrm{rel}}$, according to the law of propagation of error. In this way it is possible to evaluate whether the precision of a method is better with external calibration or with an internal standard. When use of the internal standard gives no improvement or even impairs the precision the critical steps can be found by the decoding method mentioned. If these steps can be improved or eliminated the internal standard technique should be used. If not, it is better to avoid the use of an internal standard, since it is often easier and less time-consuming to look for a suitable external calibration approach. In any case, if an assay for a drug in biological material has been developed with the use of an internal standard, it should be established that this technique does not impair the precision of the method.

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