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Selection of robust combinations of extraction liquid composition and internal standard

Monte Carlo simulation of improvement of assay methods with liquid–liquid extraction prior to high-performance liquid chromatography

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ABSTRACT

The liquid–liquid extraction of a mixture of sulphonamides was achieved to examine the correlation between the experimental errors in the recoveries. Also, the impact of the composition of the extraction liquid was investigated. Six sulphonamides were repeatedly extracted simultaneously with ten different extraction liquids and determined with a reversed-phase high-performance liquid chromatographic (HPLC) system. The means, standard deviations and covariances (or correlations) of the recoveries were calculated. These data showed that correlation between the extraction of two or more structurally related compounds depends strongly on the extraction liquid composition used: the selection of an appropriate extraction liquid is very important for the development of accurate and reproducible assay methods. Selection of improper extraction liquids may introduce errors in internal standard calibration that are larger than the errors in external standard calibration. The selection of a suitable internal standard is also very important for the development of accurate and reproducible assay methods. Even compounds that are structurally related to the analyte may demonstrate completely different extraction behaviour. Selection of a proper internal standard and an accurate extraction liquid increases the

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accuracy and precision of the method. To investigate the influence on routine analysis, the data were used to simulate 50 analytical runs (calibration graphs with quality control samples) for each sulphonamide separately with external and internal standard calibration. In the latter option the other five sulphonamides were all tested as internal standards. This was done for all extraction liquids used. The results of these simulations demonstrate great differences between different extraction liquid compositions and internal standards for a given analyte. Also the calibration method (internal or external calibration) was found to be very important. Circumstances have been observed where external standard calibration gives better analysis results than internal standard calibration. The method described here can be applied for the selection of a suitable internal standard and extraction liquid for sample preparation by liquid–liquid extraction prior to HPLC.

INTRODUCTION

In bioanalytical methods, calibration graphs constructed from standard assays in relevant biological media are always required. The goal of method development is to develop accurate measurements, through improvement of procedures, precision and calibration. In biopharmaceutical analysis, three methods of calibration are commonly used: external and internal standard methods and standard addition methods. A detailed discussion of the three methods was given by Smith and Stewart [1]. A variant of the external standard technique, the deferred standard technique, was introduced by Guillemin *et al.* [2]. In this method, the compound to be analysed is injected in pure solution, some time after injection but during the chromatographic run of the real sample.

External standard methods have the disadvantage, as compared with internal standard assays, that each step must be controlled regularly. For example, in liquid–liquid extractions followed by evaporation of part of the organic phase, the partial volume of the organic phase has to be maintained constant during an analytical run or it should be weighed for each standard and each sample. The use of an internal standard through the complete procedure eliminates these problems, as the ratio of analyte and internal standard are considered.

The standard addition method is very well suited for samples with analyte concentrations near the sensitivity limit. The method has the drawback of being a one-point determination; each sample has to be analysed at least twice. If only a few samples are to be measured, the method is well suited, but multiple-sample analytical runs may be more economically analysed using internal standard methods.

The use of internal standard techniques in bioanalytical assay methods with chromatographic determination is common practice. A number of reasons can be given for the importance of the use of internal standards. First, internal standards are used for the correction of injection volume in the case of manual high-performance liquid chromatographic (HPLC) injections and partial loop fillings. Haefelfinger [3] demonstrated that under such circumstances the precision of the volume of injection may be the limiting factor for method reproducibility and that the internal standard technique improves the reproducibility; the imprecision due to the variance of the injection volume can largely be eliminated by use of an internal standard. Haefelfinger also concluded that the use of an internal standard with automated injection with complete loop filling does not improve the precision of chromatographic methods. In fact, he stated that it was better not to use an internal standard under such conditions.

Kelly *et al.* [4] introduced a method for internal standard selection with the use of an internal standard data base and a marker solution. The place of the internal standard peak in the chromatogram was the selection criterion. The approach applies to aqueous acetonitrile eluents and is essentially independent of column manufacturer.

A second argument for the use of an internal standard in chromatographic assays is the interception of chromatographic instability and measurement variability, especially when peak heights are measured in the calibration procedure. Addition of an internal standard compensates for variance in peak heights due to retention time instability or column efficiency variance, as these variances influence both the analyte and internal standard. In assays with chromatographic system instability as the only source of imprecision, addition of internal standards prior to sample preparation is not necessary and addition of an internal standard after sample preparation but prior to injection in the chromatographic system suffices. An example of

addition of internal standard after sample preparation was given by Banno *et al.* [5]. To protect the stability of their HPLC system, Wieling *et al.* [6] developed a computer program that monitors the characteristics of the internal standard such as retention time and the peak area during an analytical run. In this manner, system instabilities can be detected. Sample preparation and injection of extracts into the system are discontinued until the adverse influences have been corrected.

This guarding routine by Wieling *et al.* also monitors the operation of the (robotic) sample preparation, being the third and probably most important basis for the use of internal standards: control of sample preparation and corrections for variances in sample preparation (variance in recovery, adsorption on glassware or variances in evaporation volumes).

Summarizing, an internal standard in chromatographic assays should meet the following requirements: its chromatographic peak is completely resolved, it elutes close to the analyte, its behaviour in any sample preparation procedure is the same as that of the analyte, it is detectable under the same conditions as the analyte, it is not present in original samples and it is stable and not subjected to any reaction except for sample preparation procedures. Usually, a compound structurally as similar as possible and with similar physico-chemical properties is selected.

This paper examines the correlation of the experimental errors of simultaneously extracted sulphonamides and introduces a method for the selection of a suitable internal standard and extraction liquid in sample preparation with liquid–liquid extraction. The recoveries of a mixture of six sulphonamides after replicated liquid–liquid extractions were determined, including the statistical parameters. These results were used to simulate calibration graphs for sulphonamides with external standard calibration and with internal standard calibration by Monte Carlo methods. In Monte Carlo methods, computer-generated observations are easily obtained as independent realizations from the theoretical population distribution, using computer random generators. The mean and the variance of a sample are estimates of the population distributions and are calculated from replicated measurements. Computer simulations can be used to make analytical predictions, including variability. The algorithms for generating recoveries and peak responses after liquid–liquid extraction prior to HPLC determination are discussed in the next section.

The extractions and simulations were done with ten ternary mixtures of three extraction solvents ($\equiv$ ten extraction liquids). The composition of the extraction liquid was the only parameter changed in the liquid–liquid extraction step. In the internal standard alternative, the remaining five sulphonamides were used as internal standards. For each sulphonamide, an internal standard and extraction liquid can be selected that give the best values for the validation criteria preferred.

THEORY

In the literature, examples were found where the investigation of the characteristics of the recovery of the internal standard was ignored [7] or where the internal standard was added to the extract after the actual extraction and before the instrumental measurement [5]. In the first example the development of the method is not complete. We think that if an internal standard is used for assay control, the properties of this compound (recovery, variance of recovery, covariance of recovery with recoveries of other compounds, retention times, resolutions, etc.) should be investigated in the same way as those of the analyte, with respect to both sample preparation and chromatography. The method in the second example can only be rationalized when the sample preparation is completely under control or when no proper internal standard has been found. A paper by Osman *et al.* [8] described the use of different internal standards for different sample matrices.

In the internal standard method applied for correction of imprecision due to sample preparation, it is generally difficult to select an internal standard that meets all the necessary requirements. These requirements find their origin in both sample preparation and determination as mentioned above: a good internal standard will correct the assay for losses of the compound of interest in the isolation steps and improve the precision of the results by compensating for random errors associated with adsorption losses, extraction yield, aliquot taking, extent of derivatization, decomposition of analyte and instrumental performance. Ideally, the sample

preparation yield of the internal standard and the compound of interest should have a constant ratio throughout the isolation and preparation steps. Therefore, the variance of recoveries of both analyte and internal standard should be completely correlated. This should also be the case in the extraction of a drug with one or more metabolites. It is economically advantageous to determine drug and metabolites within one assay method instead of analysing each compound separately, and therefore it is necessary to find suitable conditions.

In sample preparation using liquid–liquid extraction and internal standard calibration, two quantities determine the quality of the real extraction step of a compound: first, the extraction yield of the analyte (and to a lesser extent that of the internal standard) is important, as high recoveries guarantee more sensitive methods. Second, a robust ratio of the extraction yields of the analyte and the internal standard has a considerable effect on the precision and accuracy of the method (robust with respect to varying random conditions). In cases where several analytes are extracted in one extraction step (*e.g.*, a drug with several metabolites or co-drugs), all recoveries should be maximized and the robustness of all ratios of recoveries should be optimized. If the recovery of a compound is *ca.* 100%, then it can be expected that the variation in recovery will be small. However, if recoveries of 100% cannot be obtained, then one should try to optimize the variance in the recovery and in the extraction ratio, if an internal standard is used.

According to Curry and Whelpton [9] the probability that the internal standard will adversely affect the data is significant and should be considered. This can occur in a number of ways according to Curry and Whelpton: first, if an error can arise in the extraction of the analyte, then it can arise just as easily in extraction of the internal standard; second, if calibration is non-linear (*e.g.*, in gas chromatographic assays with electron-capture detection), then a variance in the internal standard response will inevitably cause, rather than correct, an error in the response to the analyte.

The first argument by Curry and Whelpton can be rejected in many instances for the following reason: if two compounds are extracted simultaneously, they are both subjected to a number of random variables that are not under control of the analyst: small variances in room temperature, small variances in the sample matrix used, small variances in glassware, etc. However, we expect that these variances will have more or less the same effect on the amount of analyte and internal standard extracted into the organic phase if their physico-chemical properties and structures are similar. In other words, the variances in the recoveries of both compounds are more or less correlated. This leads to the conclusion that, even if analyte and internal standard recoveries have large variances, an assay may be good if these variances in the recoveries of both compounds are highly correlated, that is, if random variables have identical effects on both compounds. It is the responsibility of the analytical chemist to find a suitable internal standard and a suitable composition of the extraction liquid. The combination of these two elements should provide a constant extraction ratio of internal standard and analyte. The second argument of Curry and Whelpton (non-linearity) is not a problem arising during sample preparation, but is a detection problem that can be solved by proper selection of calibration experiments and/or calibration models.

A problem in sample preparation using liquid–liquid extraction that demands the use of internal standard may be the different extraction behaviours of the internal standard and analyte with respect to variance in random conditions (room temperature, time of extraction and intensity of extraction). This may introduce errors larger than the errors in the external standard calibration. Indeed, in these instances the internal standard should be removed from the procedure, substituted by a different compound or the complete liquid–liquid extraction procedure should be modified/changed (other extraction liquid, etc.).

In two papers [10,11], Snyder and Van der Wal discussed the sources of imprecision that can affect assays based on solvent extraction and HPLC analysis. An example was given that demonstrates an increase in assay imprecision with decrease in analyte recovery. Snyder and Van der Wal also explained why internal standard calibration does not guarantee an improvement in assay precision: precision may sometimes even be worse for internal standard calibration, because the number of measurements in internal standard calibration is double that in external standard calibration. The overall

precision of assay methods may therefore sometimes be negatively affected if the imprecision in the double measurements is larger than the imprecision of external standard calibration, where no correction is made for procedure variance.

The mean recovery, R_a, and the variance of this recovery, S_a^2, of an analyte depend strongly on the sample matrix and on the composition of the extraction liquid [12–16]. Introduction of an internal standard introduces two new quantities, R_i and S_i^2. The ratio of the recoveries Q and its variance S_Q^2 are determined by the recoveries R_a and R_i of both compounds, the variances in the recoveries S_a^2 and S_i^2 and the covariance $S_{a,i}^2$ between the recoveries of both compounds when they are extracted simultaneously.

The ratio of the recoveries of two compounds is expressed by the ratio of the two recoveries:

$$Q = R_a/R_i \tag{1}$$

The rules for error propagation give an expression for the variance of the ratio of the recoveries:

$$S_Q^2 = S_a^2\left(\frac{\partial Q}{\partial R_a}\right)^2 + S_i^2\left(\frac{\partial Q}{\partial R_i}\right)^2 + 2S_{a,i}^2\left(\frac{\partial Q}{\partial R_a}\cdot\frac{\partial Q}{\partial R_i}\right)$$
$$= Q^2\left[\left(\frac{S_a}{R_a}\right)^2 + \left(\frac{S_i}{R_i}\right)^2 - 2\cdot\frac{S_{a,i}^2}{R_aR_i}\right] \tag{2}$$

The partial derivatives provide an estimate of the change in the overall response variable Q with a change in one of the component variables (R_a and R_i) while the other component variable is held constant.

When the variables R_a and R_i are completely independent of each other (*i.e.*, uncorrelated) the covariance $S_{a,i}^2$ is zero and the variance of the ratio is

$$S_Q^2 = Q^2\left[\left(\frac{S_a}{R_a}\right)^2 + \left(\frac{S_i}{R_i}\right)^2\right] = \frac{S_a^2}{R_i^2} + Q^2\cdot\frac{S_i^2}{R_i^2} \tag{3}$$

The covariance can be calculated from n replicate measurements of the recoveries of both compounds:

$$S_{a,i}^2 = \frac{\sum_{j=1}^{n}(R_{a_j} - \bar{R}_a)(R_{i_j} - \bar{R}_i)}{n-1} \tag{4}$$

The correlation coefficient ρ is a measure of the correlation between the two variances of the recovery of each compound:

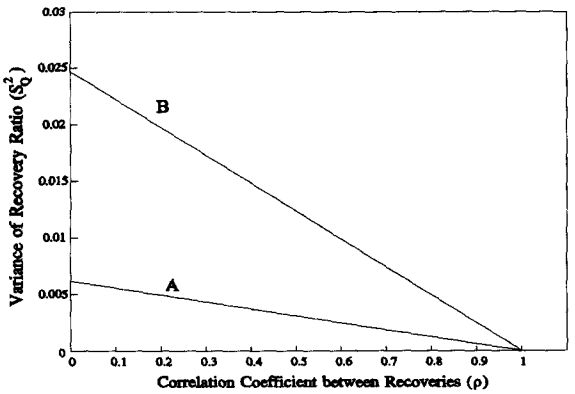

Fig. 1. Variance of the ratio of the recovery of analyte and internal standard as a function of their correlation. [$R_i = R_a = 90\%$; $S_i = S_a$ = (A) 5% and (B) 10%].

$$\rho = S_{a,i}^2/S_aS_i \tag{5}$$

A detailed discussion on error propagation was given by Ku [17] and Balke [18].

In conclusion, the variance of the ratio of the recovery of two compounds is a function of the ratio Q, the recoveries of the compounds R_a and R_i, the variances of the recoveries of both compounds S_a^2 and S_i^2 and the correlations between those variances ρ. Figs. 1 and 2 describe the influence of the variances and the correlation coefficient on the variance of the ratio Q (S_Q^2). In Fig. 1, the recoveries of both compounds are assumed to be 90% ($Q = 1$) and their standard deviations are both 5% in situation A and both 10% in situation B. The variance in the ratio of the recoveries is plotted against the correlation coefficient between the experimental errors. There is a linear relationship between correlation coefficient and the variance of the recovery ratio (Fig. 1). In Fig. 2, the variance in the ratio is plotted against the variance in the recoveries of both compounds (the S.D.s for both compounds are equal) for different correlation coefficients between the experimental errors in the recovery. There is a linear relationship between the variance in the recoveries of the analytes and the variance in the recovery ratio (Fig. 2), which can also be understood from eqn. 2. Figs. 1 and 2 and eqn. 2 show that not only the correlation of experimental errors is important for method quality, it is also important that the individual experimental errors are not too large.

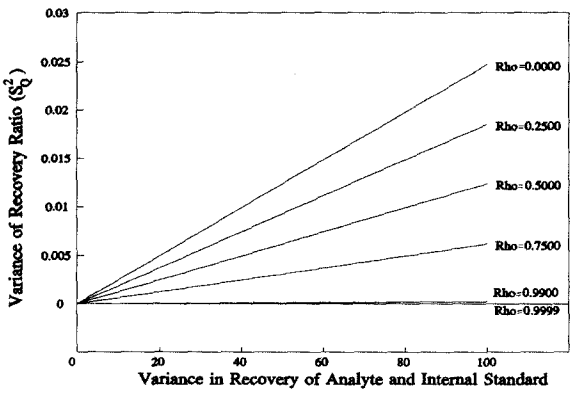

Fig. 2. Variance of the ratio of the recovery of analyte and internal standard as a function of their individual variances ($S_i = S_a$) at different correlation coefficients ($R_i = R_a = 90\%$).

EXPERIMENTAL

Instruments and instrumental conditions

The assay was performed with an HPLC system consisting of a Spectra-Physics (San Jose, CA, USA) SP8700 solvent-delivery system at a flow-rate of 1.0 ml min^{-1} and a Kratos (Ramsey, NJ, USA) Model 757 UV detector, wavelength 260 nm, range 0.005 a.u.f.s., rise time 1 s. The injections of extracts into a Zymark (Zymark, Hopkinton, MA, USA) Z 310 HPLC injection station, equipped with an electrically controlled Rheodyne valve and a 20-μl sample loop, were performed by a Zymate II robot system. The Zymark Z 310 Analytical Instrument Interface was used to control the HPLC injection station. Data analysis was performed by means of a Spectra-Physics Chromjet SP4400 computing integrator. The analytical column was a Microsphere 3-μm C_{18} cartridge system (100 × 4.6 mm I.D.) (Chrompack, Middelburg, Netherlands).

Mixing was performed on a Type VF2 vortex mixer (Janke und Kunkel, Staufen, Germany), shaking of the extraction container was performed on a Heidolph (Kelheim, Germany) Reax-2S shaker and a Labofuge GL (Heraeus-Christ, Osterode am Harz, Germany) was used for centrifugation.

Simulations and calculations were performed on an IBM PS/2 Model 80-A31 computer using a laboratory-made software package written in Pascal under MS-DOS 3.3.

Chemicals and reagents

The sulphonamides sulphisomidine (SOMI), sulphathiazole (THIA), sulphapyridine (PYRI), sulphamerazine (MERA), sulphamethoxypyridazine (MEPY) and sulphachloropyridazine (CLPY) were supplied by Sigma (St. Louis, MO, USA). The structural formulae are given in Fig. 3. Acetonitrile (ACN), tetrahydrofuran (THF), dichloromethane (DCM) and methanol (MeOH) were supplied by Labscan (Dublin, Ireland) and were of HPLC grade. Chloroform (ChromAR) (Clf) was of analytical-reagent grade and supplied by Malinckrodt (Promochem, Wesel, Germany). Acetic acid (100%) (HAc), triethylamine (TEA), phosphoric acid (85%), potassium dihydrogenphosphate (KH_2PO_4) and ammonium acetate were all of analytical-reagent grade and supplied by Merck (Darmstadt, Germany). Methyl *tert.*-butyl ether (Uvasol) (tBME) was also supplied by Merck. Water was purified by using a Milli-RO-4 and a Milli-Q water purification system

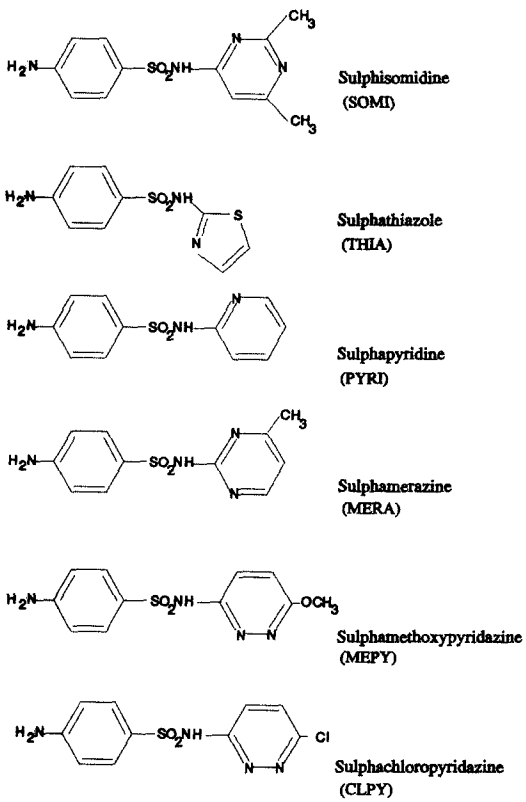

Fig. 3. Structural formulae of the sulphonamides investigated.

TABLE I

EXTRACTION LIQUID COMPOSITIONS INVESTIGATED FOR THE SIMULTANEOUS EXTRACTION OF SIX SULPHONAMIDES

No.	Fraction DCM	Fraction Clf	Fraction tBME
1	1.000	0.000	0.000
2	0.000	1.000	0.000
3	0.000	0.000	1.000
4	0.500	0.500	0.000
5	0.500	0.000	0.500
6	0.000	0.500	0.500
7	0.333	0.333	0.333
8	0.666	0.167	0.167
9	0.167	0.666	0.167
10	0.167	0.167	0.666

(Millipore, Bedford, MA, USA). Unless stated otherwise, Milli-Q-water was used.

Dichloromethane, chloroform and methyl *tert.*-butyl ether were used to prepare ten pure, binary and ternary extraction liquids in accordance with Table I and Fig. 4. These three solvents are commonly used extraction solvents and were selected from the solvent selectivity theory according to Rohrschneider [19] and Snyder [20]. The solvents selected were similar to those which Glajch *et al.* [21] used for normal-phase liquid chromatography. Methyl *tert.*-butyl ether was selected instead of diethyl ether as it is less volatile.

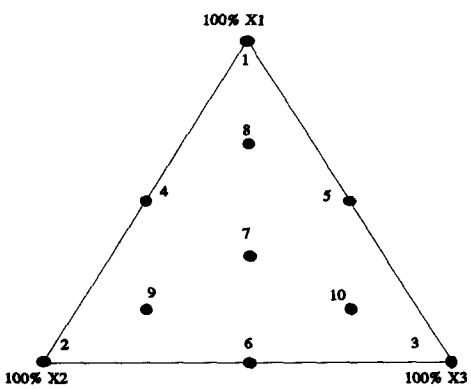

Fig. 4. Extraction liquid compositions where the recoveries of six sulphonamides have been measured prior to simulation. X1 = Dichloromethane; X2 = chloroform; X3 = methyl *tert.*-butyl ether.

An acetate buffer (pH 5.0; 0.5 *M*) was prepared by dissolving 3.85 g of ammonium acetate in 100 ml of water. pH adjustment was performed using concentrated acetic acid.

A phosphate buffer (pH 3.0; 0.05 *M*) was prepared by dissolving 6.80 g of KH_2PO_4 in 1000 ml of water. pH adjustment was performed using concentrated phosphoric acid. To this buffer 4.15 ml of TEA and 10 ml of acetic acid were added.

The mobile phase was optimized using the POEM (Predicting Optimal Eluent Mixtures) computer optimization package [22]. A description of the optimization of the mobile phase was given by Wieling *et al.* [23]. The mobile phase was prepared by mixing 1 ml of acetonitrile, 5 ml of THF and 140 ml of methanol and adding phosphate buffer (pH 3.0; 0.05 *M*) to 1000 ml.

The stock solutions of sulphonamides were prepared by dissolving 100 mg of the compounds in 100 ml of methanol. These solutions were stored at 4°C and were used to prepare a standard solution (1 mg l^{-1} in water) used for the extraction studies containing all six sulphonamides. This solution was stored at 4°C.

Analytical procedure

A 250-µl aliquot of blank human plasma, 250 µl of the standard solution and 250 µl of acetate buffer solution were pipetted in a 11.5-ml glass tube and mixed for 10 s on a vortex mixer. An aliquot of 9 ml of extraction liquid were added and the tubes were extracted for 5 min with a Heidolph tumble mixer at 20 rpm.

After centrifugation at 4000 rpm (2755 *g*) for 10 min, the organic layer was transferred to another 11.5-ml glass tube and evaporated to dryness under a gentle stream of nitrogen at 55°C. The residue of the sample was reconstituted in 1 ml of 50% methanol and 20 µl were injected into the HPLC system. Extractions were repeated five times. Peak areas were measured as the response criterion.

For the determination of the absolute analytical recovery of the sulphonamides, the peak areas of prepared samples were compared with peak areas measured by injecting directly seven times the standard solution into the HPLC system.

Calibration graph simulation

Internal standard calibration simulation. For each

composition of the extraction liquid the mean and standard deviation of the recoveries of the six sulphonamides were determined with the procedure outlined above. Also, the covariances and the correlations between the variances of the recoveries of each pair of sulphonamides were determined.

These data were used to simulate calibration graphs with eight different concentrations of analyte of 10, 20, 50, 100, 200, 500, 800 and 1000 concentration units l^{-1}. One sulphonamide was selected as the analyte, while the five other sulphonamides were successively used as internal standards. In turn each sulphonamide was selected as the analyte. The simulation of a calibration graph was always accompanied by simulation of four quality control samples (duplicates at two concentration levels, 15.00 and 900.0 concentration units l^{-1}).

Simulation of standards was done with the following procedure, which demonstrates the simulation of a calibration graph for an assay method using liquid–liquid extraction as the sample clean-up step. One particular analyte, one particular internal standard and one particular extraction liquid were used in the procedure.

Recoveries and peak areas were simulated with an algorithm that produces random data of the recoveries of internal standard according to a gaussian distribution [24] as follows.

(1) For a particular extraction liquid composition: simulate an observation of the recovery of the analyte R_a by adding a random error component S_a to the "true" value of the recovery of the analyte $\bar{R}_a$:

$$R_a = \bar{R}_a + S_a \cos[2\pi random(0\ldots1)]\{\sqrt{-2\ln[random(0\ldots1)]}\}$$

or

$$R_a = \bar{R}_a + S_a \sin[2\pi random(0\ldots1)]\{\sqrt{-2\ln[random(0\ldots1)]}\}$$

Both equations are needed alternately to obtain random results.

(2) For the same extraction liquid composition, simulate an observation of the recovery of the selected internal standard R_i by adding a random error component S_i to the "true" value of the recovery of the internal standard $\bar{R}_i$ using the correlation $\rho_{a,i}$ between the variances of the recoveries of analyte and internal standard:

$$R_i = \rho_{a,i} \cdot \frac{S_i}{S_a}(R_a - \bar{R}_a) + \bar{R}_i + \sqrt{S_i(1-\rho_{a,i}^2)} \cdot \cos[2\pi random(0\ldots1)]\{\sqrt{-2\ln[random(0\ldots1)]}\}$$

or

$$R_i = \rho_{a,i} \cdot \frac{S_i}{S_a}(R_a - \bar{R}_a) + \bar{R}_i + \sqrt{S_i(1-\rho_{a,i}^2)} \cdot \sin[2\pi random(0\ldots1)]\{\sqrt{-2\ln[random(0\ldots1)]}\}$$

Both equations are needed alternately to obtain random results.

(3) Generate a peak area for the analyte from the simulated observation R_a. If the recovery of the analyte is 100% a peak area of 100 000 was generated for the highest standard. For the lower concentration levels a peak area was generated of 100 000 times a concentration factor:

$$\textit{peak area standard} = 100\,000R_a \cdot \frac{\textit{concentration units standard}}{\textit{concentration units highest standard}}$$

(4) Generate a peak area for the internal standard from the simulated observation R_i. For the internal standard a peak area of 50 000 was generated in the case of 100% recovery:

$$\textit{peak area internal standard} = 50\,000R_i$$

(5) Repeat steps 1–4 for each concentration level of the analyte to obtain the complete calibration graph (= eight times).

(6) Simulate four quality control samples (duplicates at two concentration levels, 15.00 and 900.0 concentration units l^{-1}) using steps 1–4.

In this way, a calibration graph consisting of eight points is obtained, together with two quality control samples in duplicate. In turn each sulphonamide is considered as being the analyte; the other five sulphonamides are successively observed as being the internal standard for each extraction liquid composition in Fig. 4. The ratio of the peak area of the analyte to that of the internal standard is used as the response factor (Fig. 5).

This total procedure (steps 1–6) is repeated 50 times for each combination of analyte, internal standard and extraction liquid (= 50 analytical runs).

The procedure above denotes that the magnitude and the variance in the recovery of the analyte in this

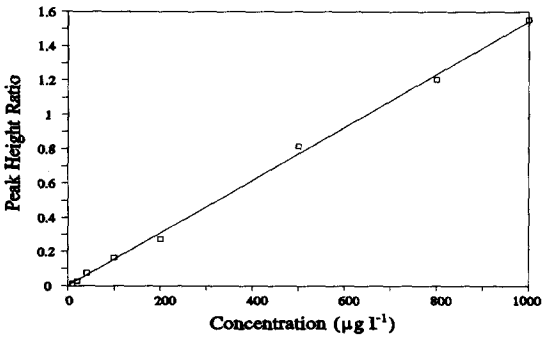

Fig. 5. Example of a simulated calibration graph at eight concentration levels.

simulation experiment are not affected by the concentration of the analyte (the relative standard deviation of the recovery is constant; no concentration effect on recovery is simulated). As a consequence, the standard deviation of the peak area ratio is proportional to concentration.

External standard calibration simulation. A similar procedure was followed for the external standard calibration, in which the simulation of internal standard recoveries and internal standard peak areas was omitted. The correlation coefficients between the experimental errors of the recoveries of two solutes were not required: steps 2 and 4 in the procedure described above are omitted. Here, the response used for calculating calibration models was the peak area of the analyte.

Procedure summary

Recovery data for the replicate extraction of a mixture of sulphonamides in ten different extraction liquid compositions were obtained. The recovery data were used to select robust combinations of internal standard and extraction liquid composition for a particular analyte. The differences in the quality of the different methods were predicted and demonstrated by Monte Carlo simulation of assay methods using liquid–liquid extraction prior to HPLC for the given analyte, internal standard and extraction liquid. Alternatively, if external standard calibration may give better quality control data, the internal standard was omitted.

It is also possible to use the method for the selection of an extraction liquid composition that gives the most robust results for all recoveries and recovery ratios after extraction of several analytes.

TABLE II

RECOVERIES AND STANDARD DEVIATIONS OF THE SULPHONAMIDES FOR THE DIFFERENT COMPOSITIONS OF THE EXTRACTION LIQUID

Fractional composition of extraction liquid			Sulphonamide						
DCM	Clf	tBME	SOMI	THIA	PYRI	MERA	MEPY	CLPY	$\overline{\text{C.V.}}$ (%)[a]
1.000	0.000	0.000	0.759 (0.016)	0.755 (0.020)	0.931 (0.017)	0.962 (0.019)	0.940 (0.012)	0.950 (0.022)	2.0
0.000	1.000	0.000	0.741 (0.027)	0.551 (0.022)	0.885 (0.032)	0.950 (0.031)	0.931 (0.028)	0.923 (0.029)	3.4
0.000	0.000	1.000	0.694 (0.032)	0.684 (0.030)	0.890 (0.032)	0.910 (0.034)	0.894 (0.039)	0.945 (0.047)	4.3
0.500	0.500	0.000	0.746 (0.013)	0.710 (0.011)	0.954 (0.008)	0.985 (0.012)	0.972 (0.007)	0.944 (0.017)	1.3
0.500	0.000	0.500	0.796 (0.015)	0.799 (0.019)	0.891 (0.017)	0.911 (0.020)	0.876 (0.018)	0.900 (0.023)	2.2
0.000	0.500	0.500	0.676 (0.017)	0.666 (0.024)	0.848 (0.026)	0.911 (0.029)	0.882 (0.030)	0.904 (0.027)	3.1
0.333	0.333	0.333	0.726 (0.017)	0.739 (0.021)	0.887 (0.022)	0.902 (0.021)	0.885 (0.023)	0.875 (0.033)	2.7
0.666	0.167	0.167	0.767 (0.016)	0.841 (0.015)	0.949 (0.015)	0.961 (0.016)	0.951 (0.012)	0.932 (0.020)	1.8
0.167	0.666	0.167	0.728 (0.032)	0.743 (0.028)	0.908 (0.033)	0.943 (0.036)	0.922 (0.028)	0.889 (0.028)	3.6
0.167	0.167	0.666	0.693 (0.008)	0.779 (0.016)	0.901 (0.020)	0.921 (0.026)	0.897 (0.024)	0.901 (0.026)	2.3
$\overline{\text{C.V.}}$ (%)[b]			2.6	2.9	2.5	2.6	2.4	3.0	2.7

[a] Mean of six coefficients of variation for one extraction liquid.
[b] Mean of ten coefficients of variation for one solute.

TABLE III

CORRELATIONS BETWEEN THE MEASUREMENT ERRORS OF THE RECOVERIES OF EACH PAIR OF SULPHONAMIDES FOR TEN DIFFERENT COMPOSITIONS OF THE EXTRACTION LIQUID

Fractional composition of extraction liquid			Sulphonamide pair					
DCM	Clf	tBME	SOMI THIA	SOMI PYRI	SOMI MERA	SOMI MEPY	SOMI CLPY	THIA PYRI
1.000	0.000	0.000	0.9171	0.9333	0.9462	0.7501	0.9312	0.9868
0.000	1.000	0.000	0.9932	*0.9785*	0.9694	0.8728	0.8756	0.9530
0.000	0.000	1.000	0.9880	0.9772	0.9784	*0.9681*	0.9763	*0.9973*
0.500	0.500	0.000	0.8170	0.6643	**−0.1801**	0.2627	0.3584	0.8720
0.500	0.000	0.500	*0.9974*	0.9713	*0.9971*	0.8299	0.6775	0.9731
0.000	0.500	0.500	0.9558	0.9550	0.9531	0.9177	0.8871	0.9943
0.333	0.333	0.333	0.9501	0.9013	0.9151	0.9537	*0.9898*	0.9291
0.666	0.167	0.167	**0.4097**	0.7716	0.7348	0.6188	0.4885	**0.5801**
0.167	0.666	0.167	0.9559	0.9517	0.9509	0.8969	0.7154	0.9899
0.167	0.167	0.666	0.6877	**0.1562**	0.1186	**−0.2302**	**−0.4712**	0.8138
Minimum correlation			0.4097	0.1562	−0.1801	−0.2302	−0.4712	0.5801
Maximum correlation			0.9974	0.9785	0.9971	0.9681	0.9898	0.9973

TABLE IV

RATIOS OF THE RECOVERIES (Q) AND THEIR STANDARD DEVIATIONS (S_Q) OF EACH PAIR OF SULPHONAMIDES FOR TEN DIFFERENT COMPOSITIONS OF THE EXTRACTION LIQUID

Fractional composition of extraction liquid			Sulphonamide pair					
DCM	Clf	tBME	SOMI THIA	SOMI PYRI	SOMI MERA	SOMI MEPY	SOMI CLPY	THIA PYRI
1.000	0.000	0.000	1.006	0.816	0.789	0.808	0.799	0.811
			(0.011)	(0.007)	(0.006)	(0.012)	(0.007)	(0.007)
0.000	1.000	0.000	1.344	0.837	0.780	0.795	0.802	0.623
			(0.008)	(0.006)	(0.007)	(0.014)	(0.014)	(0.008)
0.000	0.000	1.000	1.015	0.780	0.763	0.777	0.734	0.768
			(0.007)	(0.010)	(0.009)	(0.009)	(0.008)	(0.007)
0.500	0.500	0.000	1.052	0.782	0.758	0.768	0.790	0.744
			(0.010)	(0.010)	(0.017)	(0.013)	(0.015)	(0.007)
0.500	0.000	0.500	0.997	0.894	0.874	0.909	0.885	0.897
			(0.005)	(0.004)	(0.004)	(0.011)	(0.016)	(0.006)
0.000	0.500	0.500	1.014	0.797	0.742	0.766	0.748	0.786
			(0.015)	(0.008)	(0.008)	(0.011)	(0.010)	(0.006)
0.333	0.333	0.333	0.982	0.818	0.804	0.820	0.830	0.833
			(0.010)	(0.009)	(0.008)	(0.006)	(0.012)	(0.009)
0.666	0.167	0.167	0.912	0.808	0.798	0.807	0.823	0.886
			(0.020)	(0.011)	(0.012)	(0.014)	(0.018)	(0.014)
0.167	0.666	0.167	0.979	0.802	0.772	0.789	0.819	0.819
			(0.013)	(0.012)	(0.011)	(0.017)	(0.025)	(0.004)
0.167	0.167	0.666	0.890	0.769	0.752	0.773	0.769	0.864
			(0.013)	(0.018)	(0.021)	(0.024)	(0.028)	(0.011)
C.V. (%)[b]			1.1	1.2	1.3	1.6	1.9	1.0

[a] Means of the coefficient of variation of the ratio of the recoveries of two compounds of fifteen combinations of two compounds in one particular extraction liquid composition.

[b] Means of the coefficient of variation of the ratio of the recoveries of two compounds in ten different extraction liquid compositions.

THIA MERA	THIA MEPY	THIA CLPY	PYRI MERA	PYRI MEPY	PYRI CLPY	MERA MEPY	MERA CLPY	MEPY CLPY
0.9773	0.9457	0.7661	0.9825	0.8989	0.8308	0.8652	0.8082	0.5517
0.9375	0.8368	0.8179	0.9884	0.9214	0.9207	0.8709	0.9624	0.8040
0.9910	0.9800	*0.9971*	0.9820	0.9674	*0.9952*	*0.9952*	*0.9949*	*0.9853*
0.3039	**0.5341**	0.8129	**0.5762**	**0.7582**	0.8554	**0.5049**	**0.7164**	0.7760
0.9951	0.8497	0.6560	0.9760	0.8168	0.7467	0.7969	0.7222	**0.2270**
0.9966	*0.9845*	0.8186	0.9915	*0.9937*	0.8669	0.9789	0.8216	0.8451
0.9477	0.9533	0.9138	0.9975	0.9838	0.9138	0.9907	0.9197	0.9581
0.5385	0.5582	0.7356	0.9955	0.9537	0.9008	0.9736	0.8918	0.8786
0.9991	0.9627	0.8889	0.9943	0.9879	0.8673	0.9732	0.8933	0.8843
0.7905	0.5437	**0.2890**	*0.9992*	0.9235	**0.7045**	0.9367	0.7227	0.8969
0.3039	0.5341	0.2890	0.5762	0.7582	0.7045	0.5049	0.7164	0.2270
0.9991	0.9845	0.9971	0.9992	0.9937	0.9952	0.9952	0.9949	0.9853

THIA MERA	THIA MEPY	THIA CLPY	PYRI MERA	PYRI MEPY	PYRI CLPY	MERA MEPY	MERA CLPY	MEPY CLPY	$\overline{\text{C.V.}}$ (%)[a]
0.784	0.803	0.795	0.967	0.990	0.980	1.024	1.013	0.990	
(0.006)	(0.012)	(0.014)	(0.004)	(0.009)	(0.013)	(0.011)	(0.014)	(0.019)	1.1
0.580	0.592	0.597	0.931	0.950	0.958	1.020	1.029	1.008	
(0.009)	(0.013)	(0.014)	(0.006)	(0.014)	(0.014)	(0.016)	(0.009)	(0.019)	1.4
0.752	0.765	0.724	0.978	0.996	0.942	1.018	0.963	0.946	
(0.007)	(0.007)	(0.005)	(0.007)	(0.012)	(0.014)	(0.007)	(0.013)	(0.010)	1.0
0.720	0.730	0.752	0.968	0.981	1.010	1.013	1.043	1.030	
(0.012)	(0.010)	(0.008)	(0.010)	(0.005)	(0.011)	(0.011)	(0.013)	(0.013)	1.3
0.877	0.912	0.888	0.978	1.017	0.990	1.039	1.012	0.974	
(0.002)	(0.012)	(0.018)	(0.005)	(0.012)	(0.017)	(0.015)	(0.018)	(0.028)	1.2
0.731	0.755	0.737	0.931	0.962	0.938	1.033	1.008	0.976	
(0.004)	(0.005)	(0.016)	(0.004)	(0.005)	(0.015)	(0.007)	(0.019)	(0.018)	1.2
0.819	0.835	0.845	0.983	1.003	1.015	1.020	1.032	1.012	
(0.008)	(0.008)	(0.013)	(0.002)	(0.005)	(0.018)	(0.004)	(0.019)	(0.014)	1.1
0.875	0.884	0.903	0.987	0.998	1.019	1.011	1.032	1.021	
(0.015)	(0.014)	(0.013)	(0.002)	(0.005)	(0.010)	(0.005)	(0.010)	(0.012)	1.3
0.788	0.806	0.836	0.963	0.984	1.022	1.022	1.061	1.038	
(0.001)	(0.009)	(0.015)	(0.004)	(0.008)	(0.018)	(0.011)	(0.018)	(0.015)	1.3
0.845	0.868	0.864	0.978	1.005	1.000	1.027	1.022	0.995	
(0.014)	(0.020)	(0.026)	(0.005)	(0.010)	(0.021)	(0.010)	(0.021)	(0.013)	2.0
1.0	1.4	1.8	0.5	0.9	1.5	0.9	1.5	1.6	1.3

RESULTS AND DISCUSSION

Results of the extraction

The recoveries of the sulphonamides and their standard deviations after extraction into the ten extraction liquids are given in Table II. Also, mean coefficients of variation (C.V.) are given for each extraction liquid composition, demonstrating the differences between the variance of extraction of each composition. As the extractions were repeated five times, there is some uncertainty in the resulting variance and covariance estimates. A discussion on confidence limits for population variance was given by Box *et al.* [25]. We assume that the recoveries measured ($R_1, R_2, \ldots, R_n$) are independent, normally distributed random variables having mean μ and variance σ^2. The standardized sum of squares, $\sum z_j^2$, of deviations from the population means has a chi-squared distribution with n degrees of freedom:

$$\sum z_j^2 = \frac{\sum (R_j - \mu)^2}{\sigma^2} \sim \chi^2$$

For very precise estimation of a population variance many observations are needed. This is beyond the scope of this paper; here, it is important to obtain a first estimate of variances and covariances.

The influence of the extraction liquid composition (C.V. = 1.3–4.3%) on the variance of the extraction of individual compounds is far more significant than the influence of the solute (C.V. = 2.4–3.0%). A Bartlett test [26] indicated that the variance in the direction of extraction liquid compositions was significantly different from the variance in the direction of solutes. The correlations between the experimental errors of the recoveries of each pair of sulphonamides in each extraction liquid are given in Table III. The highest values for the standard deviations in the recoveries are measured in pure chloroform and methyl *tert.*-butyl ether (Table II). However, especially in pure methyl *tert.*-butyl ether, correlations between the experimental errors are very high. This high correlation reduces the standard deviation of the ratio of the recoveries in this extraction liquid composition. Small variance in the recoveries is measured in binary (50:50) mixtures of dichloromethane and chloroform. However, here the correlations are significantly lower than those for the previous extraction liquids. Table IV gives the values for Q and S_Q for all pairs of sulphonamides. The differences in the variance of the extraction of the compounds that could be seen in the direction of extraction liquids (Table II) are considerably reduced when it concerns ratios of recoveries. Table IV shows that in the direction of compounds (C.V. = 0.9–1.9%) and in the direction of extraction liquids (C.V. = 1.0–2.0%), the robustness is approximately equal. The overall mean coefficient of variation of ratios of recoveries is 1.3%, which is less than 50% of that of separate recovery data (overall mean coefficient of variation = 2.7%). Hence, generally, ratios of the extraction yield of two compounds are more robust than individual recovery data. A clear exception to this statement is presented by sulphisomidine and sulphachloropyridazine in extraction liquid 10: the coefficients of variation of individual recoveries are 1.2% and 2.9%, respectively, whereas the coefficient of variation of their ratio is 3.6%. From Table IV, the differences between the use of different extraction liquid compositions is clear: in the extraction of sulphisomidine with sulphamerazine used as the internal standard, the coefficient of variation in the ratios of the recoveries are as follows:

extraction liquid 2: C.V. = 0.007/0.780 · 100 = 0.9%

extraction liquid 3: C.V. = 0.009/0.763 · 100 = 1.2%

extraction liquid 4: C.V. = 0.017/0.758 · 100 = 2.2%

extraction liquid 10: C.V. = 0.021/0.752 · 100 = 2.8%

From all tables, a clear effect of the composition of the extraction liquid can be seen. Recoveries and standard deviations of recoveries and correlations of the recoveries of pairs of sulphonamides vary with the extraction liquid composition.

The combination of analyte and internal standard with the largest range between the minimum and maximum correlation between the experimental errors of the recoveries over the different extraction liquids is the combination of sulphisomidine with sulphachloropyridazine. This combination also demonstrates the worst correlation of all combinations (−0.4712 in extraction liquid composition 10, Table III). Under these conditions, the standard deviation of the ratio of the recoveries is 3.6% (0.028/0.769 · 100%). Sulphapyridine and sulpha-

methoxypyridazine demonstrate the smallest range. The best correlation is demonstrated by the recoveries of sulphapyridine and sulphamerazine with a ternary mixture (extraction liquid 10). Under these conditions, the coefficients of variation of the recoveries of these sulphonamides are 2.2% and 2.9%, respectively. The C.V. of their ratio is small, 0.5%. Most stable values for the C.V. of the ratio of recoveries are measured for sulphapyridine and sulphamerazine in extraction liquids 7 and 8 (0.2%). From the results discussed above, it can be concluded that optimization of extraction liquid composition in addition to the choice of the internal standard is possible.

Monte Carlo simulation of routine analysis of sulphisomidine

As the results for sulphisomidine and sulphachloropyridazine show the largest differences, this pair is used to demonstrate the internal standard and extraction liquid composition selection procedure by means of routine analysis simulation with Monte

TABLE V

SUMMARY OF SIMULATED CALIBRATION GRAPHS FOR SULPHISOMIDINE WITH EXTERNAL STANDARD CALIBRATION AND WITH SULPHACHLOROPYRIDAZINE AS THE INTERNAL STANDARD FOR DIFFERENT EXTRACTION LIQUIDS

$\mu(x_1) = 15.00$; $\mu(x_2) = 900.0$; $n = 50$.

Extraction liquid composition[a]	$\bar{r}$[b] (range)	$\bar{x}_1$[c] (range)	C.V. (%) (range)	$\bar{x}_2$[c] (range)	C.V. (%) (range)
1[d]	0.99971	14.44	1.8	899.1	1.5
	0.99886–1.0000	10.56–18.50	0.2–5.4	861.0–940.2	0.1–5.4
1	0.99996	15.08	0.6	898.5	0.7
	0.99980–1.0000	13.58–16.82	0.0–2.5	881.7–913.8	0.0–2.1
2[d]	0.99931	15.44	3.1	899.8	3.0
	0.99806–0.99998	4.26–23.88	0.0–9.4	809.0–992.7	0.0–9.1
2	0.99986	15.37	1.2	895.28	1.3
	0.99947–0.99999	11.92–18.10	0.0–4.3	869.2–924.8	0.0–3.4
3[d]	0.99887	16.05	4.0	897.77	4.4
	0.99291–0.99995	3.81–23.83	0.1–23.8	830.2–986.1	0.1–11.7
3	0.99993	14.90	1.0	898.30	0.9
	0.99966–0.99999	13.33–16.42	0.0–2.5	881.3–923.5	0.1–2.4
4	0.99978	15.08	1.4	901.12	1.5
	0.99906–0.99999	9.42–19.72	0.0–4.6	861.2–948.6	0.0–4.8
5	0.99982	15.12	1.6	898.96	1.4
	0.99878–0.99999	10.15–18.95	0.2–4.2	869.2–933.9	0.0–3.5
6	0.99990	14.90	1.2	900.30	1.0
	0.99949–1.0000	11.81–17.56	0.1–3.7	872.0–923.6	0.0–4.5
7	0.99985	15.12	1.2	896.31	1.1
	0.99941–0.99999	12.07–18.19	0.0–4.2	876.8–923.8	0.0–3.3
8	0.99981	15.14	1.8	901.36	2.0
	0.99927–1.0000	10.24–19.02	0.1–5.0	866.5–947.3	0.0–5.5
9	0.99946	15.20	2.7	899.89	2.9
	0.99788–0.99999	9.91–22.88	0.0–8.6	860.2–992.5	0.0–8.4
10[d]	0.99992	15.03	1.0	899.58	0.9
	0.99959–1.00000	12.89–18.05	0.0–3.0	876.4–922.2	0.1–3.0
10	0.99935	15.40	2.7	899.5	3.3
	0.99750–0.99994	7.01–20.49	0.0–9.7	829.1–963.4	0.0–8.0

[a] For compositions, see Table I.
[b] r = Correlation coefficient of the calibration graph simulated.
[c] $\bar{x}_i$ = Mean concentration of a quality control sample analysed in duplicate with a coefficient of variation C.V. (%).
[d] Simulation of external standard calibration.

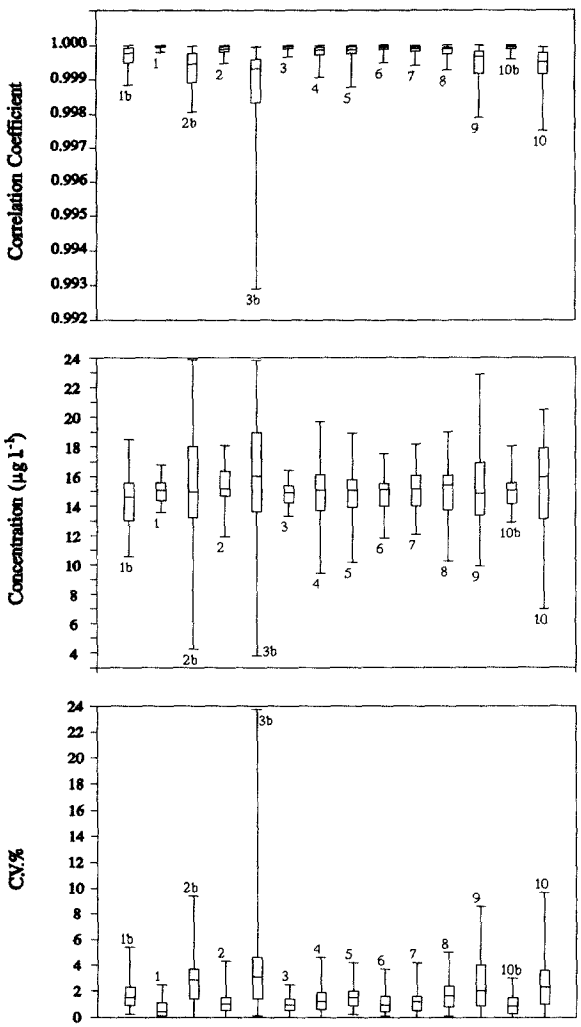

Fig. 6. Box-and-whisker plots for the correlation coefficients of the calibration graphs, the mean of quality control sample (μ = 15.00 μg l^{-1}) and the coefficient of variation of the duplicate quality control samples (μ = 15.00 μg l^{-1}) after simulation of the extraction of sulphisomidine with internal and external standard calibration using different extraction liquid compositions (data in Table V).

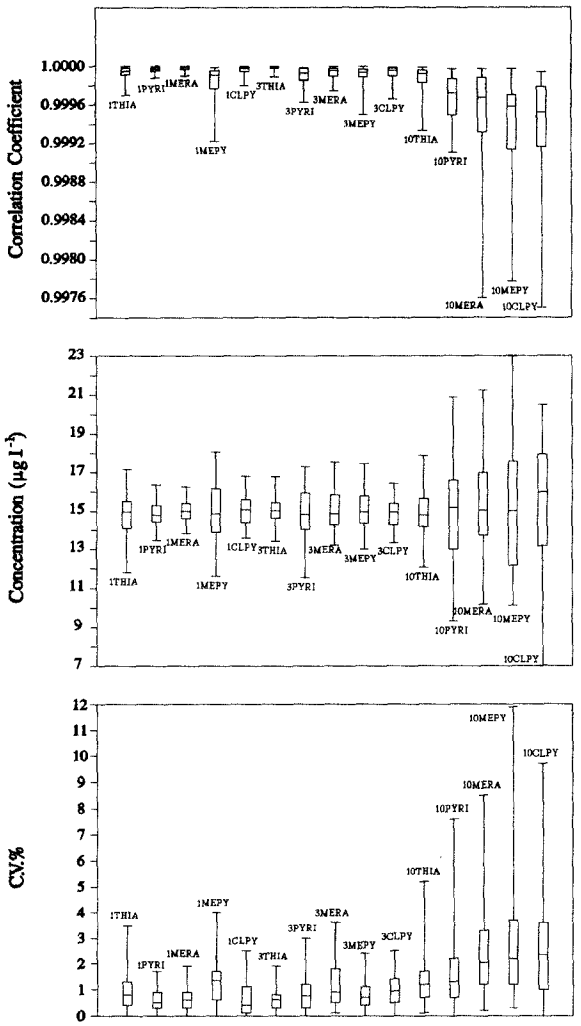

Fig. 7. Box-and-whisker plots for the correlation coefficients of the calibration graphs, the mean of quality control sample (μ = 15.00 μg l^{-1}) and the coefficient of variation of the duplicate quality control samples (μ = 15.00 μg l^{-1}) after simulation of the extraction of sulphisomidine with different extraction liquid compositions and different internal standards (data in Table VI).

Carlo methods. A summary of the calibration graphs simulated for sulphisomidine and sulphachloropyridazine with different extraction liquids is given in Table V. The data in this table are also plotted as box-and-whisker plots in Fig. 6 to illustrate the differences graphically. The central boxes in the plots cover the middle 50% of the data values, between the lower and upper quartiles. The whiskers extend out to the minimum and maximum values, while the central lines are at the medians. Table VI and the box-and-whisker plots in Fig. 7 show the simulation results of liquid–liquid extraction of sulphisomidine with five internal standards in three extraction liquids. These tables and figures demon-

TABLE VI

SUMMARY OF SIMULATED CALIBRATION GRAPHS FOR SULPHISOMIDINE WITH FIVE OTHER SULPHONAMIDES AS INTERNAL STANDARD AND WITH THREE DIFFERENT EXTRACTION LIQUIDS

$\mu(x_1) = 15.00$; $\mu(x_2) = 900.0$; $n = 50$.

Extraction liquid[a]	Internal standard	$\bar{r}^b$ (range)	$\bar{x}_1{}^c$ (range)	C.V. (%) (range)	$\bar{x}_2{}^c$ (range)	C.V. (%) (range)
1	THIA	0.99993	14.79	1.0	901.00	0.9
		0.99970–1.0000	11.81–17.19	0.0–3.5	879.8–919.8	0.0–3.0
	PYRI	0.99997	14.86	0.6	899.70	0.6
		0.99988–1.00000	13.46–16.35	0.0–1.7	884.9–915.9	0.0–1.9
	MERA	0.99998	15.03	0.6	899.36	0.7
		0.99990–1.0000	13.83–16.26	0.0–1.9	886.5–913.3	0.0–2.4
	MEPY	0.99984	14.97	1.4	901.87	1.3
		0.99922–0.99999	11.61–18.09	0.0–4.0	885.9–928.7	0.0–3.9
	CLPY	0.99996	15.08	0.6	898.5	0.7
		0.99980–1.0000	13.58–16.82	0.0–2.5	881.7–913.8	0.0–2.1
3	THIA	0.99998	15.01	0.6	900.54	0.6
		0.99989–1.0000	13.41–16.80	0.0–1.9	884.6–913.9	0.0–1.9
	PYRI	0.99989	14.93	1.0	898.86	0.9
		0.99963–0.99999	11.54–17.30	0.0–3.0	876.2–928.9	0.0–2.5
	MERA	0.99994	15.11	1.2	899.06	0.9
		0.99975–1.0000	13.20–17.54	0.1–3.6	867.2–922.9	0.0–2.9
	MEPY	0.99990	15.12	0.8	898.69	1.0
		0.99950–1.0000	12.99–17.46	0.0–2.4	878.9–919.3	0.0–2.5
	CLPY	0.99993	14.90	1.0	898.30	0.9
		0.99966–0.99999	13.33–16.42	0.0–2.5	881.3–923.5	0.1–2.4
10	THIA	0.99985	14.85	1.4	900.00	1.1
		0.99933–0.99999	12.05–17.88	0.1–5.2	870.1–926.2	0.1–3.0
	PYRI	0.99966	15.02	1.6	897.11	1.6
		0.99910–0.99997	9.31–20.89	0.0–7.6	849.4–944.5	0.0–4.9
	MERA	0.99948	15.16	2.5	896.17	2.7
		0.99760–0.99997	10.16–21.24	0.2–8.5	822.6–944.2	0.0–8.2
	MEPY	0.99940	14.98	2.9	896.67	2.5
		0.99777–0.99997	10.12–22.96	0.3–11.9	847.4–958.0	0.0–7.5
	CLPY	0.99935	15.40	2.7	899.5	3.3
		0.99750–0.99994	7.01–20.49	0.0–9.7	829.1–963.4	0.0–8.0

[a] For compositions, see Table I.

[b] r = Correlation coefficient of the calibration graph simulated.

[c] $\bar{x}_i$ = Mean concentration of a quality control sample analysed in duplicate with a coefficient of variation C.V. (%).

strate that the quality of a method can be improved by selection of the proper internal standard and with extraction by a suitable extraction liquid. Criteria to judge these improvements here are the predictive qualities of the calibration model (concentration of control samples) and reproducibility (C.V.) of the concentrations of control samples and the linearity of the simulated calibration graphs (correlation coefficient r).

The results in Tables V and VI are based on recovery data. Errors arising during phase separation, evaporation, injection, etc., are not taken into account in this simulation experiment: they are assumed to be equal for all extraction liquid compositions. Concluding, the only source of variation assumed here that causes variation in experimental error and in covariation of experimental errors is the extraction liquid composition and the potential internal standard selected. The results of the simulations (especially with external standard calibration)

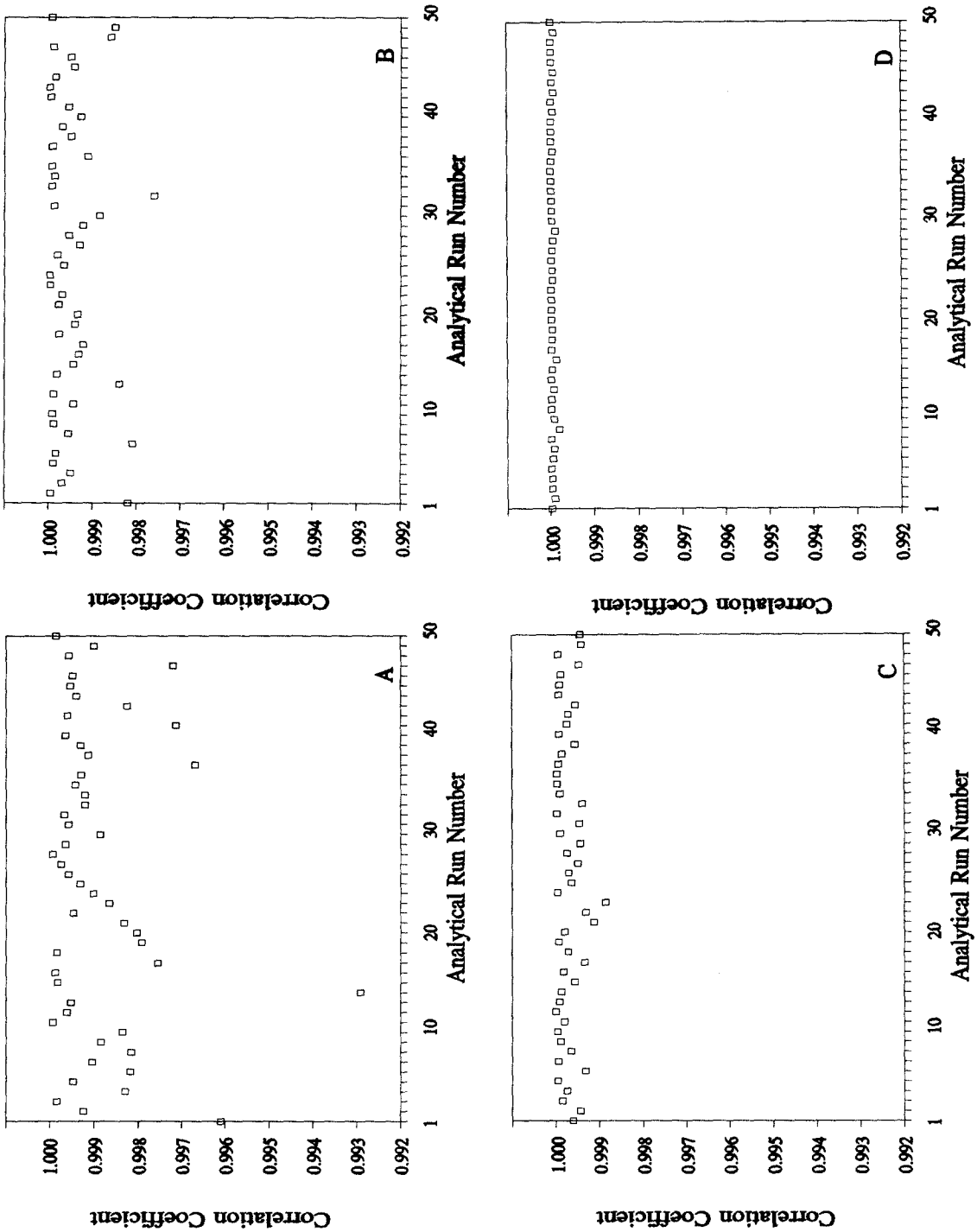

Fig. 8. Quality control chart of linearity of 50 simulated calibration graphs using four different combinations of internal standard and extraction liquid. For description of methods A, B, C and D, see Results and Discussion.

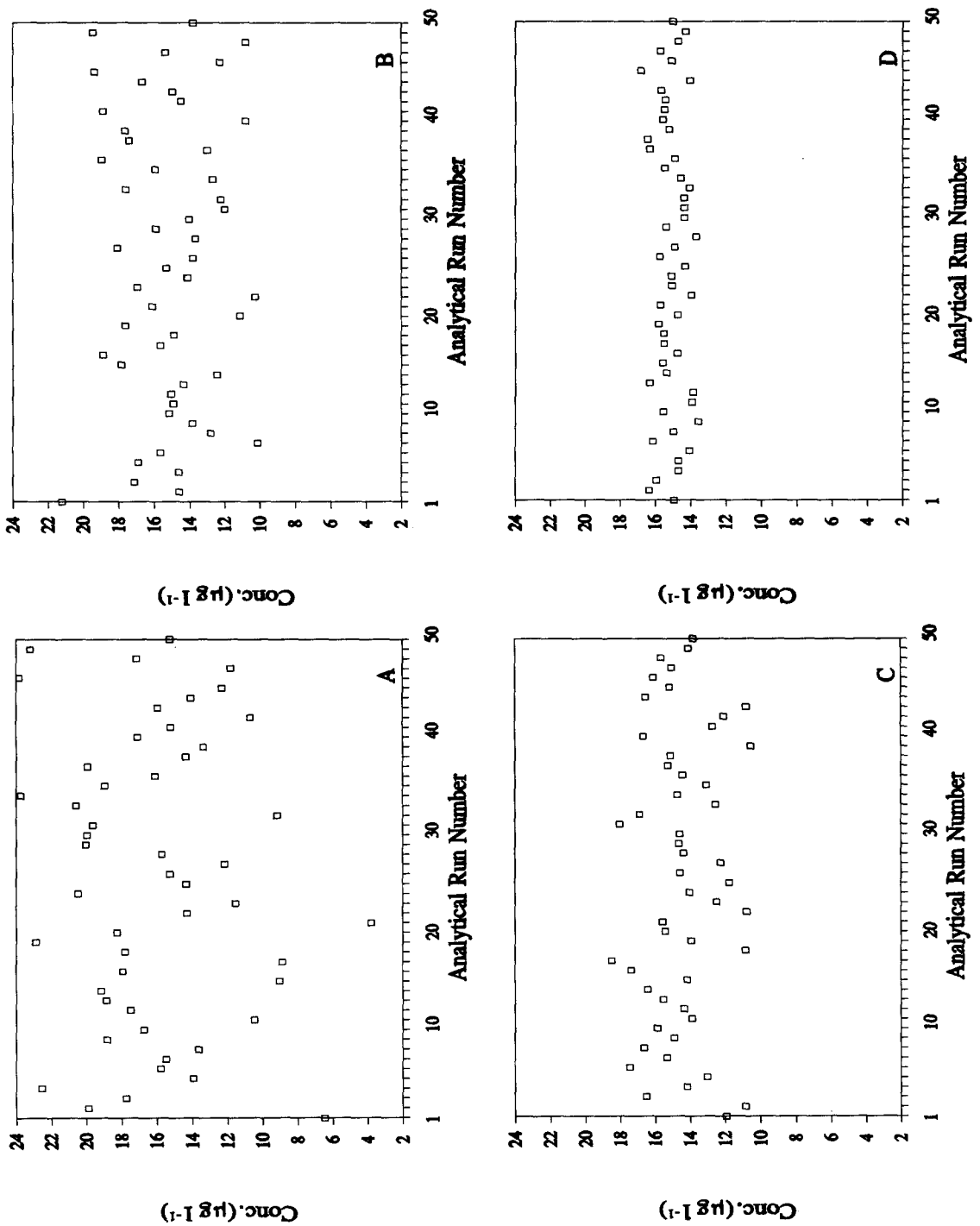

Fig. 9. Quality control chart of control samples (concentrations; $n = 2$) during 50 simulated analytical runs using four different combinations of internal standard and extraction liquid ($\mu = 15.00\ \mu g\ l^{-1}$). For description of methods A, B, C and D, see Results and Discussion.

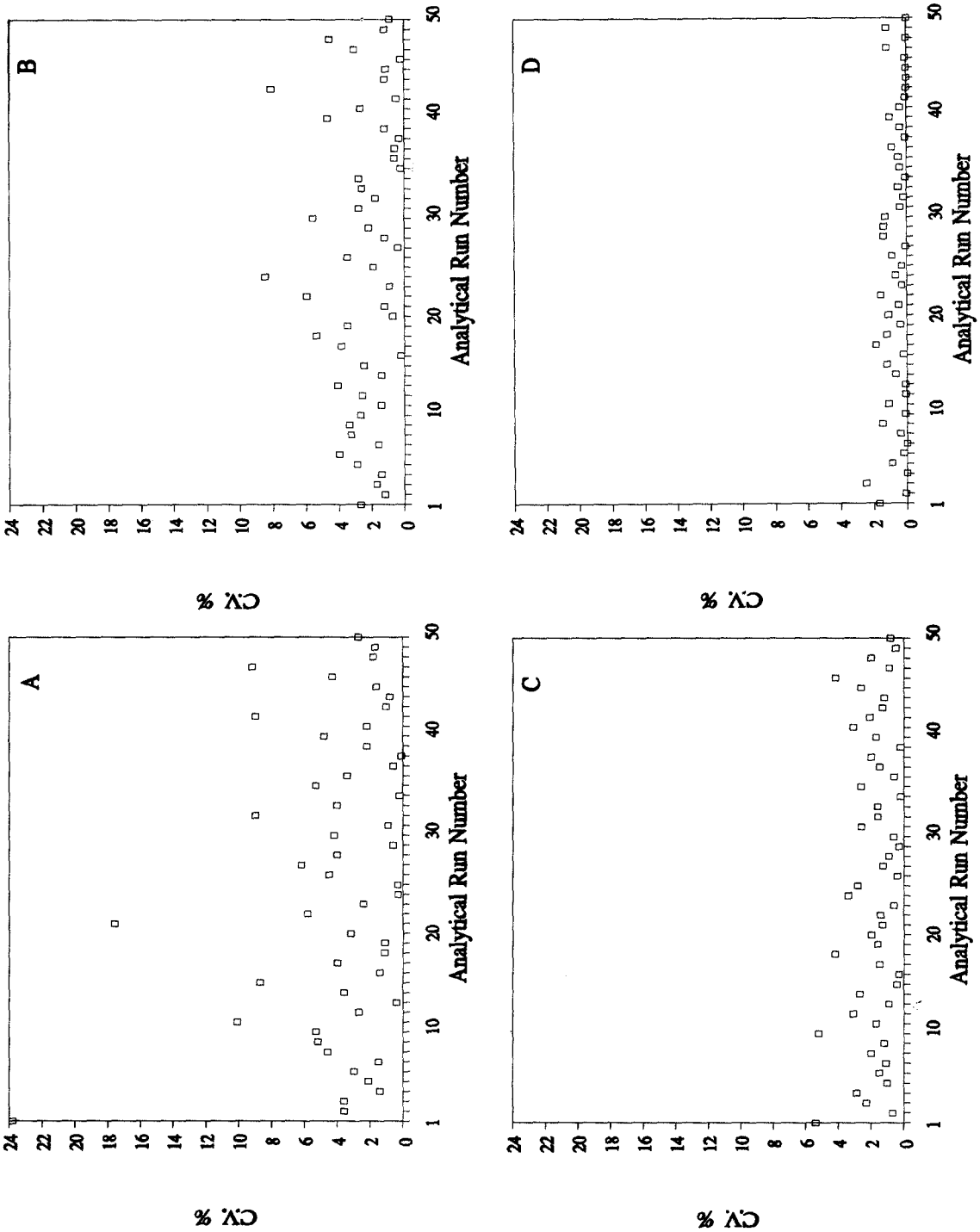

Fig. 10. Quality control chart of control samples (coefficient of variation of duplicates of $\mu = 15.00\ \mu g\ l^{-1}$) during 50 simulated analytical runs using four different combinations of internal standard and extraction liquid. For description of methods A, B, C and D, see Results and Discussion.

may be slightly optimistic as compared with the results one might obtain during the actual routine analysis: there may be small concentration effects, especially when low concentrations are used. However, the results of the routine analysis simulation give a good initial estimate of the behaviour under laboratory circumstances.

From Table V, it can be observed that there is a very large difference between external and internal standard calibration, especially for extraction into extraction liquid 3. An exception to this statement is extraction liquid composition 10. If sulphisomidine is being extracted with this composition it is better not to use the internal standard method, as better results are obtained with external standard calibration: none of the sulphonamides used in this investigation is suitable as an internal standard with extraction liquid composition 10 (Table VI). Figs. 8–10 demonstrate the simulated routine analysis (50 analytical runs) of sulphisomidine under four different conditions. These figures illustrate the difference that may arise between different methods with respect to calibration method, internal standard selected and extraction liquid composition selected. Situation (A) represents analysis with external standard calibration using extraction liquid composition 3. Situation (B) represents analysis with internal standard (sulphamerazine) calibration using extraction liquid composition 10. Situation (C) represents analysis with external standard using extraction liquid composition 1. Situation (D) represents analysis with internal standard (sulphachloropyridazine) using extraction liquid composition 1. It is clear that situation (A) is inferior to situation (D) for the analysis of sulphisomidine: linearity of the calibration graphs, C.V. of the duplicate quality control samples and predicted mean concentrations of duplicate quality control samples are much better. External standard calibration in extraction liquid composition 1 (C) is better than internal calibration with sulphamerazine in extraction liquid composition 10 (B).

Table II demonstrates that the standard deviation of the recoveries are relatively small (maximum C.V. *ca.* 5%). Much greater variances of the ratios of recoveries may be obtained from extractions with relatively large S.D. values.

The extraction procedure of the mixture of sulphonamides from plasma by replicate measurements from ten different extraction liquid compositions is accomplished within 24 h. The simulation of each combination of analyte, internal standard and extraction liquid composition takes *ca.* 12 h of calculation on the IBM PS/2 Model 80-A31 computer.

It can be calculated that this simulation includes 18 000 analytical runs, *i.e.*, 216 000 analyses. This represents *ca.* 15 years of experimental work [5 days per week, five analytical runs = $(5 \cdot 8) + (5 \cdot 4) = 60$ analyses in 24 h], which is impossible to accomplish.

CONCLUSIONS

Experimental errors in the recoveries of structurally related compounds are more or less correlated. However, the extraction liquid chosen greatly affects the correlation between the errors in the recovery of analyte and internal standard. The selection of an appropriate extraction liquid is very important for the development of accurate and reproducible assay methods. Selection of unsuitable extraction liquids may introduce errors in internal standard calibration that are larger than errors in external standard calibration.

Also, the choice of the internal standard is very important: even compounds that are structurally related to the analyte may demonstrate a dissimilar extraction behaviour. It is well reasoned to select as the internal standard a stucturally related compound that demonstrates an extraction behaviour in the selected extraction liquid which is most similar.

Generally, internal standard calibration gives better results for liquid–liquid extraction than external standard calibration. However, circumstances can be indicated where external standard calibration is better.

A method has been developed for the selection of an extraction liquid and/or an internal standard in liquid–liquid extraction sample preparation prior to HPLC analysis. The quality of routine analysis is used as a selection criterion. This quality is approximated by simulation of 50 analytical runs under different conditions (extraction liquid composition and calibration method, Figs. 8–10). The quality control results under these conditions are compared to give optimum extraction conditions.

The method developed may also be very useful for the selection of the composition of an extraction

liquid that gives the most robust results for all recoveries and recovery ratios after extraction of several analytes.

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