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A VALIDATION PROCESS FOR DATA FROM THE ANALYSIS OF DRUGS IN BIOLOGICAL FLUIDS

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INTRODUCTION

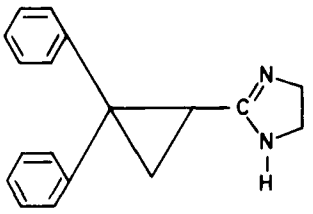
The Federal Good Laboratory Practice (GLP) regulations of June 1979 for non-clinical laboratory studies and the impending Good Clinical Practice (GCP) regulations require that all analytical data from pivotal studies included in an IND/NDA submission meet specific criteria for acceptability. In order to ensure compliance, a quality assurance unit was established in the Department of Drug Metabolism at Hoffmann-La Roche, Nutley, NJ whose responsibility was to prepare requirements for the acceptability of data generated from bioanalytical procedures, and to ensure that the requirements are met (validation). Based upon the above charter the quality assurance unit designed an assay validation process for analytical data treatment, data reduction and data reporting. This validation process, which has been in place approximately 3 years, is currently used to monitor the analytical data being generated by the various Roche facilities and clinical and contract laboratories worldwide for compounds at various stages of drug development.

0363-9045/85/1109-1703$3.50/0

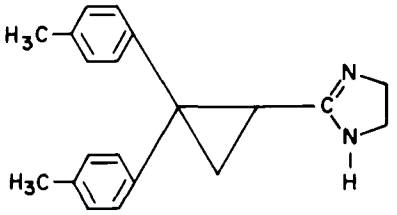
The assay validation process can be subdivided into three distinct phases. The first two phases are assay development in the research laboratory, and the transfer of the assay from the development laboratory to the applications laboratory for "set-up". These two phases constitute the "In-Vitro" segment of assay validation in which the assay is defined and no experimental samples are assayed. The distinction between the assay development laboratory and the assay application laboratory is very important in that quite often the group performing the routine assays are not the group that developed the assay. The application laboratory is quite often at another location with differing instrumentation and level of expertise. The validation process therefore requires re-validation in the application laboratory under a "new" set of experimental or laboratory conditions prior to the assay of the experimental samples. The third phase is the actual analysis of the toxicological or clinical samples, and is defined as the "In-Vivo" segment of assay validation.

The assay development and application validation processes can be demonstrated with the experimental drug Cipralan (cibenzoline succinate, I, Figure 1) which is currently under development as a cardiac antiarrhymic agent.

The assay (1) involves the extraction of the compound into benzene from plasma buffered to pH 11, and the HPLC analysis of the residue. A 10 μM sulfonate ion-exchange column was used with an acetonitrile:phosphate buffer (0.015 M, pH 6.0) (80:20) as the mobile phase. UV detection at 214 nm was used for quantitation with the di-para-methyl analogue of I ([II], Figure 1) as the internal standard.



[I]



[II]

Figure 1. Chemical Structure of Cibenzoline [I] and di-p-methylcibenzoline (internal standard, [II]).

A. Assay Development and Transfer

The assay development phase of the validation process consists of a detailed written description by the analytical method development laboratory of the assay along with the "In-Vitro" validation document (Table 1) and supportive data. The departmental QA unit then reviews the submission and approves or rejects the assay procedure. Approval of the assay indicates that the assay is validated for the analysis of experimental samples by the development laboratory or for transfer of the method to another application laboratory for "set-up" prior to the assay of experimental samples.

The "In-Vitro" validation documentation is a brief, concise one page summary of all the critical analytical parameters related to the assay. It should be noted that separate "In-Vitro" assay validation documents are required for each drug and metabolite(s) in each type of biological fluid (e.g., blood, plasma or urine) and in each specie (e.g., man, dog or rat).

Table 1. "In-Vitro" Validation Document

(Assay Development Laboratory) *
(Application Laboratory)**

DRUG ADMINISTERED	CLINICAL INDICATION	
Ro 22-7796/001 Cipralan	Antiarrhythmic	
ANALYTICAL STANDARD	LOT NO	SOURCE
Ro 22-7796/001	9147-82-2	A. Laurenzano
INTERNAL STANDARD	LOT NO	SOURCE
Ro 22-7937/001	LE-4146	UPSA

Test Species: Man **Biological Medium:** Plasma

Collection Device. Vacutainer tubes **Anticoagulant/Preservative:** Heparin

Type of Assay: HPLC/UV at 214 nm **Reference:** Hackman et al. J. Chromatogr. 273 (1983) 347-356

Measurement Method. Peak height/area: Ratio **Regression Method:** Power

Intra-Assay Precision: Conc. Range. 2.5-1000 ng/ml n*= 3 Precision: ± 4.48 % (C.V.)

Inter-Assay Precision: Conc. Range: 2.5-1000 ng/ml n*= 3.5 Precision: ± 6.38 % (C.V.)

Overall % Recovery of Assay: 78.6 ± 11.8 (15.0% CV) Conc. Range: 2.5-1000 ng/ml

Sensitivity Limit Validated: 5.0 ng/ml using 1.0 ml Precision: ± 3.29% (C.V.)

Specificity: Parent Drug Only/Major Metabolite(s): Ro 22-7796 specific against Ro 23-0264/000, Ro 23-0809/001, Ro 23-8995/000, Ro 23-0607/001 and o-methyl catechol metabolite (89-9119-3)

Sample Storage Stability. Temp: -17°C Duration: 3 months Result: Stable

ANALYST	ASSAY LABORATORY (LOCATION)	DATE
D. Sandor	Roche, Bldg #86, Rm. 742	2/8/84
LABORATORY NOTEBOOK REFERENCES	SUPERVISOR	
D.S. No. 12994 pp. 3-12	Mr. C. V. Puglisi	

Q A OFFICER	DATE	APPROVED	REJECTED
	2/14/84	X	

REASON FOR REJECTION

* To be submitted with written assay procedure
** To be submitted prior to the analysis of unknown samples

F 9676* (REV 11/82)

The uppermost portion of the form details the compounds used for the analytical investigation. The Ro number is the Roche numerical designation of the compound with the first six digits identifying the drug compound (Ro 22-7796 is the number for cibenzoline) and the three digit number following the slash (001) identifying the form of the compound (the succinate salt). Lot numbers and sources are required to monitor and document the purity and stability of the compounds over the long time-course of the pre-clinical and clinical phases of drug development.

The next portion of the form describes the specie, fluid collected, type of collection device and anticoagulant (where appropriate). For the assay of cibenzoline the "In-Vitro" validation form was submitted for human plasma collection using heparinized vacutainers. Also listed on this portion of the form is the assay reference [Hackman et. al., J. Chromatogr. (1983)], and the type of assay (HPLC/UV detection at 214 nm). The method of measurement (peak height ratio of the response of cibenzoline to the response of the internal standard versus the concentration of cibenzoline), and the regression method (power) used for the least squares regression analysis of the calibration data are also described.

In order to insure consistency in data accumulation, treatment and reduction, a glossary of "standard" analytical terms was established. Recovered (spiked or processed) standards are defined as the concentrations of authentic standards added to control (drug-free) biological specimens which are processed to construct the calibration curve for quantitation of experimental samples.

The Range of Quantitation is defined as the highest to lowest concentration of recovered standards. Data falling outside this

range is considered invalid and requires: (a) dilution or reassay of a smaller aliquot (if above range) or (b) assay of a larger volume (if below range) with re-validation of the new calibration range. This strict definition allows no data to be reported which falls outside of the range of quantitation, and thus outside the range of validation.

Accuracy is by far the most difficult parameter to assess in establishing a new analytical method. Generally, samples of known concentration are difficult to prepare synthetically. Comparison of data obtained from prior analyses of the same samples by other reference methods will aid in the assessment of accuracy. However these comparisons require information pertaining to the stability of the samples upon storage for the interval between the two assays as well as data pertaining to the stability of these samples upon repeated freezing and defrosting. Accuracy is typically assessed by re-fitting the ratios from the calibration standards into the regression equation derived from the calibration data, and comparing the "amount found" to the "amount added". The experimental "fit" of the data points to the regression line will also aid in the choice of the mode of regression. For data in the range of one order of magnitude (i.e., 10-100 ng/ml) linear regression will usually suffice and given an excellent fit of the data. If the range of quantitation exceeds one order of magnitude a weighted (typically 1/Y) or a linear equation power ($Y = mX^b$) equation will almost always give the better fits due to the more equitable distribution of error on a relative basis rather than an absolute basis. Recent experience has indicated that the weighted regression mode be selected over the power fit to yield an equation with a

value for an intercept. The literature does report examples of using a quadratic equation for a regression analysis (2). Non-linear calibration curves are often necessary for GC/MS analysis to correct for isotope contributions of the analyte to the internal standard. Lastly, logit-log relations are used routinely for immuno-assays.

Precision by comparison is not as difficult to assess as accuracy. Precision data can be reported in terms of a coefficient of variation (C.V.) over the range of quantitation for a single experiment in which the standards are assayed in replicate (Intra-assay or Intra-day), or for a series of experiments in which the standards are assayed in singlicate over several or many experiments (Inter-assay or Inter-day). In these experiments, the "amounts found" by re-fitting the ratios from the calibration standards into the derived regression equation are evaluated. In those cases in which experimental parameters do not change over the course of multiple experiments, statistical evaluations can also be accomplished on the slope values of the regression equation. It should be noted that changes in slope may not be indicative of a precision problem. An example of this type of situation would arise as a result of a change in response factor of the analyte and not the internal standard in a separation due to column aging or deterioration. Coefficients of correlation (R) of the regression equations should be routinely calculated to evaluate the fit of the calibration data to the regression equation. Lastly, the evaluation of precision by repeated assay of samples of known concentration (Quality Control samples) is extremely useful to assess the long term precision of the method for the measurement of concentrations in the experimental samples. However, during

Table 2. Intra-Assay Precision for the Analysis of Cibenzoline in Human Plasma

STUDY: 1/25/84 INTRA-ASSAY STDS PWR

Y= .0019101 *X^ 1.006112

COEFFICIENT OF DETERMINATION (R^2): .99915
COEFFICIENT OF CORRELATION (R): .9995749

ADDED	FOUND	RATIO	RATIO/CONC.	% DEVIATION
2.500	3.0885	.00594	.00238	23.54055
2.500	2.4362	.00468	.00187	-2.55192
2.500	2.2842	.00439	.00175	-8.63368
5.000	5.0525	.00975	.00195	1.05078
5.000	4.8506	.00935	.00187	-2.98827
5.000	5.1788	.00999	.00200	3.57646
10.000	9.9953	.01936	.00194	-.04731
10.000	9.9989	.01937	.00194	-.01060
10.000	9.5111	.01842	.00184	-4.88938
25.000	24.0472	.04683	.00187	-3.81123
25.000	23.2944	.04536	.00181	-6.82259
25.000	23.6277	.04601	.00184	-5.48914
50.000	53.1447	.10401	.00208	6.28931
50.000	48.4112	.09469	.00189	-3.17758
50.000	49.5460	.09692	.00194	-.90792
100.000	98.4076	.19331	.00193	-1.59236
100.000	100.4034	.19726	.00197	.40343
100.000	97.3688	.19126	.00191	-2.63116
200.000	205.1405	.40479	.00202	2.57025
200.000	206.2547	.40701	.00204	3.12738
200.000	217.7917	.42992	.00215	8.89587
500.000	501.0833	.99418	.00199	.21666
500.000	516.4624	1.02488	.00205	3.29248
500.000	480.9820	.95406	.00191	-3.80360
1000.000	1025.8578	2.04429	.00204	2.58578
1000.000	970.2516	1.93282	.00193	-2.97484
1000.000	992.8066	1.97803	.00198	-.71934

AVERAGE % DEVIATION = 3.948144

NO. OF SAMPLES	AMOUNT ADDED	AMOUNT FOUND	+/- STANDARD DEVIATION	% COEF. OF VARIATION
3	2.50	2.60	.43	16.42
3	5.00	5.03	.17	3.29
3	10.00	9.84	.28	2.85
3	25.00	23.66	.38	1.59
3	50.00	50.37	2.47	4.91
3	100.00	98.73	1.54	1.56
3	200.00	209.73	7.00	3.34
3	500.00	499.51	17.79	3.56
3	1000.00	996.31	27.97	2.81

AVE = 4.48

assay development typically an insufficient number of experiments are performed to obtain quality control precision estimates.

Table 2 demonstrates the Intra-assay precision experiment for cibenzoline in plasma over the range of quantitation of 2.5-1000 ng/ml. Each experimental recovery standard concentration was assayed in triplicate at nine different concentrations for a total of 27 analyses. The power regression equation for this experiment was $Y = .019101 X^{1.006}$ with a coefficient of correlation of 0.99957. The closeness of the exponent of the regression equation to 1.00 demonstrates the lack of curvature of the regression equation. Using the derived regression equation, the experimental ratios at each concentration were substituted into the regression equation to yield the "amounts found". The percentage deviation at each concentration was calculated from:

$$\frac{\text{Amount Found - Amount Added}}{\text{Amount Added}} \times 100$$

The average percentage deviation from theoretical was 3.94%.

The data from the intra-assay precision experiment is summarized at each concentration to yield "amounts found" ± standard deviations (S.D.) and coefficients of variation (C.V.) at each concentration (Table 2). The average C.V. of 4.48% is reported as the mean Intra-assay coefficient of variation on the validation document (Table 1). The high C.V. at the lowest concentration is typical and due to the imprecision in measurement of peak heights at low signal to noise ratios at the limit of quantitation for the assay.

Table 3. Inter-Assay Precision for the Analysis of Cibenzoline in Human Plasma ("In-Vitro")

INTER ASSAY STATISTICS FOR BCS 00406 MAN PL 7796
CALCULATION METHOD PWR

NUMBER OF RUNS	AMOUNT 7796 ADDED (NG/ML)	AMOUNT 7796 FOUND (NG/ML)	+/-STANDARD DEVIATION	COEF. OF VARIATION(%)
5	2.500	2.646	0.322	12.157
3	5.000	4.752	0.321	6.746
3	10.000	9.935	0.987	9.935
3	25.000	23.865	1.239	5.191
3	50.000	49.361	3.538	7.168
3	100.000	99.950	3.538	3.539
3	200.000	202.679	4.414	2.178
3	500.000	513.637	32.463	6.320
3	1000.000	1002.716	42.212	4.210
			AVE =	6.383

The Inter-assay (Inter-day) precision experiment for cibenzoline in plasma was conducted over the concentration range of 2.5-1000 ng/ml (Table 3). The analyses were performed on three consecutive days over the nine point calibration range. On two days an additional 2.5 ng/ml standard was also added. Regression equations on the three days showed excellent agreement. The correlation coefficients were all greater than 0.999. The Inter-assay precision reported over this concentration range was 6.38% and this value is reported on the "In-Vitro" validation document.

Copies of the Intra- and Inter-assay precision statistics are attached to the "In-Vitro" validation document as supportive data.

The recovery of the assay which is defined as the detector response to pure authentic standards compared to the response from

Table 4. Recovery Data for the Analysis of Cibenzoline in Human Plasma

Conc. added (ng/ml)	Recovered standards mean peak height	External standards mean peak height	% Recovery
2.5	1075.0*	1809.0	89.1
5.0	2044.3	4326.0	70.9
10.0	4299.8	8861.3	72.8
25.0	12447.0	21656.0	86.2
50.0	19263.2	45857.3	63.0
100.0	44865.2	94966.0	70.9
200.0	112095.0	165560.7	101.6
500.0	231184.3	471020.0	73.6
1000.0	487127.2	925323.0	79.0
		mean =	78.6
		S.D. =	11.8
		% CV =	15.0%

*Corrected for dilution factor of 1.5

Results based on the analysis of six recovered and three external standards at each concentration, respectively.

equivalent amounts added to and recovered (corrected for aliquot) from biological specimens is also reported on the "In-Vitro" assay validation document (Table 1). In general, the higher the percent recovery of the assay the greater the precision of the assay and the lower the limit of quantitation.

Table 4 shows the recovery of cibenzoline from plasma over the range of quantitation of 2.5-1000 ng/ml. The recoveries demonstrate a wide range from 63.0 to 101.6% which is not suprising based on the strong basic nature of the molecule (pKa = 10.5), and the tendency of the compound to adhere to glass surfaces. The

overall C.V. for the recovery is 15.0% versus the 4 to 6% for the Intra-and Inter-assay precision experiments, respectively. This is a striking example which demonstrates the need for an internal standard, e.g., the di-para-analogue of cibenzoline, which chemically behaves similarly to cibenzoline, and thus "normalizes" the erratic recoveries to yield an excellent precision for the analysis.

The next section of the "In-Vitro" validation document reports the limit of quantitation, specificity of the assay and the stability of the compound under storage conditions.

The sensitivity limit (limit of quantitation) is defined as the statistically validated concentration below which no data is valid or reported. The definition does not set sensitivity limits such as 3 times noise, or a specific acceptable C.V. at the lowest concentration. It should be noted that for the assay of cibenzoline the reported limit of quantitation is 5.0 ng/ml with a C.V. of 3.29%, rather than 2.5 ng/ml with a C.V. of 16.4% which was reported in the Intra-assay precision study.

The specificity of the assay must be demonstrated by the ability of the assay to measure the analyte in the presence of: (a) endogenous substances in the biological sample, (b) known metabolites of the analyte and (c) co-administered drugs and their metabolites. For most pharmacokinetic studies co-administered medications are not a problem unless drug concentration measurements are being performed in Phase 3 efficacy trials in which patients are likely to be co-medicated. Specificity for the assay of cibenzoline was demonstrated in the presence of the imidazole, hydroxylated and O-methyl catechol metabolites (Table 1).

The drug was found to be completely stable for a period of three months at -17°C (Table 1). In addition, stability data for the drug at room temperature is usually described. This information is provided to the clinical laboratory to alert the laboratory if any special handling or storage conditions are required for the drug plasma samples. Accumulation of stability data for periods of time exceeding three months is usually the responsibility of the application laboratory.

The final section of the "In-Vitro" validation document pertains to the assay documentation. The form requires that the following information be provided: name of the analyst preparing the document, the location of the laboratory work, the laboratory notebook reference, the analyst supervisor's name (and initials) and the date submitted. The bottom-most section is for the signature and the date of approval for the document.

Once the assay has been approved in the developmental laboratory it may then be used for the analysis of experimental samples. However, if the assay is to be transferred to an applications laboratory other than the development laboratory, the applications laboratory is required to re-validate the procedure in its laboratory. The validation package submitted by the applications laboratory will include a copy of the assay procedure (along with documented modifications, if required) and an "In-Vitro" form as submitted by the developmental laboratory. The QA unit will compare the submission of the applications laboratory to that of the development laboratory and will approve the validation document if similar data is obtained to that originally reported by the development laboratory. Approval

of the applications laboratory validation document will then authorize the initiation of the assay of the experimental samples.

B. Analysis of Experimental Samples

Concentration-time data obtained for the experimental samples covered under the GLP's and the proposed GCP's are monitored through the validation process. Toxicological samples usually include 4 week range finding, 4 or 13 week tolerance studies, and/or 6, 12 or 24 month tolerance studies in a variety of animal species which may include the dog, rat, mouse, rabbit and baboon. Human clinical samples are usually generated from the following studies: single and multiple dose tolerance, pilot bioavailability, dose proportionality, effect of age, food, liver or renal disease on the pharmacokinetics of the drug, metabolism studies, pharmacokinetic studies in patients and final bioavailability studies.

The results of these toxicological and clinical analyses are summarized in an analytical report which includes the "In-Vivo" validation documents and supportive data, reassay lists, and tables of concentration-time data for the experimental samples. This report is reviewed by the QA unit and after approval is released to the responsible scientist for pharmacokinetic/statistical evaluation and interpretation.

The first section of the "In-Vivo" Document (Table 5) is similar to the "In-Vitro" document and reports the identification of the material dosed, the analytical and internal standards (lot numbers and source). In addition the Roche clinical protocol number (No. 2667A) is identified and the name of the investigator physician

Table 5. "In-Vivo" Validation Document

(To be attached to tables of inter-assay precision, QC samples, repeat analysis lists and biological data)*

DRUG ADMINISTERED: Ro 22-7796/001 (Cipralan)	PROTOCOL NO: 2667A	INVESTIGATOR: Dr. Hanigan	DEPT STUDY NO: BCS 390
ANALYTICAL STANDARD: Ro 22-7796/000		LOT NO: 001051	SOURCE: TDD
INTERNAL STANDARD: Ro 22-7937/000		LOT NO: LE-4146	SOURCE: UPSA
Test Species: Man		Biological Medium: Plasma	
Collection Device: Vacutainers		Anticoagulant/Preservative: Heparin	
Date Received from Sample Co-ordinator: 7/27/83		Storage Temperature (Assay Laboratory): -17°C	
Storage Dates (Assay Laboratory): 7/27/83-Date		Storage Location (Assay Laboratory): Bldg 86/7	
Assay Dates: 7/28/83 - 11/3/83		Dates Returned to Sample Co-ordinator: - - -	

Type of Assay: HPLC/UV-214 nm

Reference: Hackman et al. J. Chromatogr.273(1983)347-356

Measurement Method: *Peak height/area*: Ratio

Regression Method: Power

Inter-Assay Precision: Conc. Range 5-1000ng/ml n** = Precision: ± 4.8 % (C.V.)

Overall % Recovery of Assay: 55-75% Conc. Range: 5-1000 ng/ml

Sensitivity Limit Validated at: 5 ng/ml using 1ml. Precision: ± 7.0 % (C.V.)

Specificity: Parent Drug Only/Major Metabolite(s): Ro 22-7796 specific against Ro 23-0264/000, Ro 23-0899/001, Ro 22-8895/000, Ro 23-0607/001 and Ro 23-4095

Quality Control Analysis:	Conc. Added	Conc. Found ± S.D. (% C.V.)	n**	% of Theoretical
		46.5 ± 2.7 ng/ml (5.8%)	26	-
		766 ± 28 ng/ml (3.7%)	26	-
Pooled "Unknown" Sample:	–			—

Sample Storage Stability: *Temp.* -17°C *Duration* 3 months *Result* Stable (see attached)

ANALYST: A. Szuna	ASSAY LABORATORY LOCATION: Bldg. 86/Rm. 744	DATE: 12/2/83
LABORATORY NOTEBOOK REFERENCES: Book 11679, p. 109-115, 117-125	SUPERVISOR: Dr. F.-J. Leinweber	
Q.A OFFICER: [signature]	DATE: 12/8/83	APPROVED ☒ REJECTED ☐
REASON FOR REJECTION		

* To be submitted with analysis of unknown samples.

** Number of determinations.

F 9877* (REV 10/84)

(Dr. Hanigan) who conducted the study. The departmental study number (BCS 390), an internal departmental identification number, is also listed.

The sample history section documents the sample type (human plasma) and collection devices (heparinized vacutainers). In addition sample tracking information is provided in regard to the date of sample receipt in the applications laboratory, the storage location and temperature prior to assay. The exact assay dates and date of sample return are also recorded. For the study listed the samples were received 7/27/83 and kept at -17°C in Bldg. 86/7th floor until completion of analysis on 11/3/83. At the time of this report the samples had not yet been returned to the sample co-ordinator. The importance of accurately tracking this data can not be over-emphasized. It is a SOP requirement that all assays for a given compound be completed from the time of collection within the fixed period of time for which stability data is available.

The assay parameter section is similar to that reported in the "In-Vitro" validation document. This section gives the type of assay, reference, measurement and regression methods. Precision data for this study were reported over the range of quantitation of 5-1000 ng/ml over the analysis period of four months. A total of thirteen analytical experiments were performed over this interval with an average coefficient of variation of 4.8% (Table 6). The regression equations and correlation coefficients are also presented (Table 7). The correlation coefficients had a mean of 0.9995 with a range of 0.9973 to 0.9999.

The recovery, sensitivity and specificity statements for the assay are similar to those reported on the "In-Vitro" validation

Table 6. Inter-Assay Precision for the Analysis of Cibenzoline in Human Plasma ("In-Vivo")

inter assay statistics for BCS 00390 MAN PL 7796
calculation method PWR

number of runs	amount 7796 added (NG/ML)	amount 7796 found (NG/ML)	+/-standard deviation	coef. of variation(%)
13	5.000	4.821	0.338	7.013
13	10.000	10.090	0.540	5.347
13	20.000	20.690	2.238	10.816
13	50.000	50.631	2.265	4.473
13	100.000	99.431	1.602	1.611
13	200.000	203.744	6.206	3.046
13	500.000	503.786	19.282	3.827
13	1000.000	971.672	23.856	2.455
			ave =	4.824

Table 7. Summary of Regression Equations for the Analysis of Cibenzoline in Human Plasma

BCS 00390 TR01 MAN PL 7796 S01	y= 0.154E-02*x^ 0.103E+01	r= 0.999959E+00
BCS 00390 TR01 MAN PL 7796 S02	y= 0.211E-02*x^ 0.982E+00	r= 0.999693E+00
BCS 00390 TR01 MAN PL 7796 S03	y= 0.231E-02*x^ 0.968E+00	r= 0.999726E+00
BCS 00390 TR01 MAN PL 7796 S04	y= 0.206E-02*x^ 0.991E+00	r= 0.999854E+00
BCS 00390 TR01 MAN PL 7796 S05	y= 0.150E-02*x^ 0.102E+01	r= 0.999759E+00
BCS 00390 TR01 MAN PL 7796 S06	y= 0.166E-02*x^ 0.102E+01	r= 0.999957E+00
BCS 00390 TR01 MAN PL 7796 S07	y= 0.172E-02*x^ 0.102E+01	r= 0.999423E+00
BCS 00390 TR01 MAN PL 7796 S08	y= 0.151E-02*x^ 0.103E+01	r= 0.999780E+00
BCS 00390 TR01 MAN PL 7796 S09	y= 0.157E-02*x^ 0.103E+01	r= 0.997326E+00
BCS 00390 TR01 MAN PL 7796 S10	y= 0.145E-02*x^ 0.104E+01	r= 0.999661E+00
BCS 00390 TR01 MAN PL 7796 S11	y= 0.133E-02*x^ 0.106E+01	r= 0.998999E+00
BCS 00390 TR01 MAN PL 7796 S12	y= 0.140E-02*x^ 0.105E+01	r= 0.999629E+00
BCS 00390 TR01 MAN PL 7796 S16	y= 0.159E-02*x^ 0.103E+01	r= 0.999769E+00

document. The recovery for this set of experiments ranged from 55-75% over the concentration range of 5-1000 ng/ml. The coefficient of variation at the lowest calibration concentration of 5.0 ng/ml was 7.0%.

Quality control samples are assayed in replicate in parallel with the recovery standards and experimental samples. These samples are the best indicator of the precision of the measurement of the experimental samples. Guidelines for the rejection of experimental data based on QC sample results are established by the applications laboratory prior to the assay of experimental samples. Quality control samples can be prepared from a pool of previously assayed experimental samples or from a synthetically fortified pool prepared by adding a fixed amount of drug to control (drug-free) biological material. The advantage of a pool from previously assayed experimental samples is that this pool is more representative of the experimental samples under assay. This pool will contain metabolites and other endogenous substances which may not be present in the synthetic sample. The disadvantage of using the previously assayed samples as a pool is that the initial concentration of this pool must be experimentally determined rather than being exactly known as is the case with the synthetic pool. For the above Cipralan study, two QC samples were prepared from a previously assayed clinical study. Each of the two samples was assayed in duplicate with each of the thirteen analytical experiments for a total of 26 assays at each concentration. For the "QC-H" (high concentration) and "QC-L" (low concentration) the amounts found were 766.4 ± 28.2 and 46.5 ± 2.7 ng/ml, respectively, with coefficients of variation of

Table 8. Summary of the Analysis of Cibenzoline in the Quality Control Sample Pools

amount 7796 Found (ng/ml)

treatment /set	date analyzed	sample pools QC-H	QC-L
TR01/S01	07/28/83	733.00	46.30
TR01/S01	07/28/83	762.00	50.70
TR01/S02	08/02/83	735.00	43.80
TR01/S02	08/02/83	749.00	46.50
TR01/S03	08/03/83	765.00	41.90
TR01/S03	08/03/83	756.00	42.00
TR01/S04	08/04/83	774.00	42.20
TR01/S04	08/04/83	744.00	43.40
TR01/S05	08/25/83	771.00	47.70
TR01/S05	08/25/83	755.00	46.90
TR01/S06	08/26/83	732.00	44.30
TR01/S06	08/26/83	772.00	46.10
TR01/S07	08/29/83	803.00	45.60
TR01/S07	08/29/83	764.00	46.40
TR01/S08	08/31/83	817.00	49.20
TR01/S08	08/31/83	780.00	50.40
TR01/S09	10/10/83	764.00	45.20
TR01/S09	10/10/83	760.00	46.30
TR01/S10	10/11/83	773.00	47.30
TR01/S10	10/11/83	787.00	48.50
TR01/S11	10/13/83	715.00	49.30
TR01/S11	10/13/83	751.00	48.00
TR01/S12	10/17/83	740.00	48.00
TR01/S12	10/17/83	805.00	52.20
TR01/S16	11/03/83	841.00	46.20
TR01/S16	11/03/83	779.00	44.90
mean		766.42	46.53
s.d.		28.18	2.67
c.v.		3.68	5.74

3.7% and 5.7%, respectively (Table 8). This data demonstrates that excellent precision was obtained for the thirteen analytical experiments over the four month assay interval.

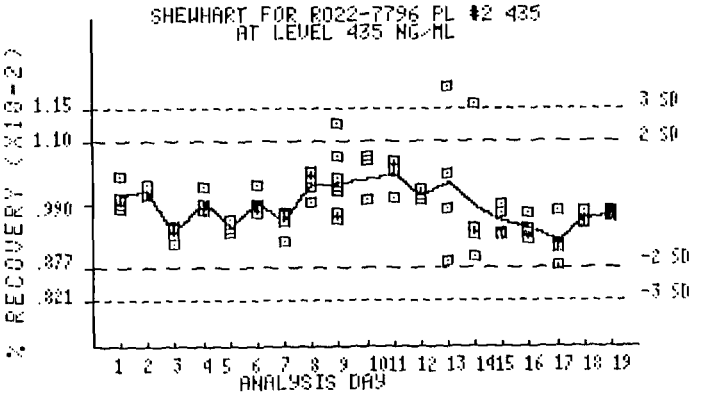


Figure 2. Shewart Quality Control Plot for the Analysis of Cibenzoline in Human Plasma.

The data can also be presented as a Shewart Plot to demonstrate the precision of the data (Figure 2). With this graphical output the mean value of the quality control sample is assigned a value as a percentage of the "amount added" and the precision of the experimental quality control samples are represented as percentages of the "amount added". The values of 2 and 3 standard deviation are also denoted to yield an excellent visual presentation of the precision of the data.

The next section of the "In-Vivo" form summarizes the stability of the compound under the conditions of storage.

The last section of the document requires that the following information be provided: the name of the analyst, supervisor, date and location of the assays and appropriate laboratory notebook reference. The bottom-most section contains the signature and date of approval for release for pharmacokinetic evaluation and interpretation.

Table 9. Typical Concentration-Time Data for the Analysis of Cibenzoline in Human Plasma

drug: R022-7796

protocol: BCS 01390 TR01 MAN PL 7796

species: MAN

dose: 160 mg

sample: PLASMA

time (HOUR)	concentration (NG/ML)			
	033M	034M	035M	036M
0.00	nm	nm	nm	nm
0.50	74.00	111.00	6.28	55.10
1.00	659.00	350.00	314.00	197.00
1.50	668.00	577.00	396.00	464.00
2.00	589.00	626.00	461.00	352.00
2.50	566.00	672.00	488.00	336.00
3.00	533.00	635.00	416.00	312.00
4.00	446.00	545.00	381.00	276.00
6.00	313.00	386.00	320.00	234.00
8.00	257.00	315.00	251.00	201.00
10.00	185.00	227.00	229.00	155.00
12.00	150.00	165.00	124.00	122.00
18.00	83.10	99.40	83.70	69.20
24.00	53.00	60.80	57.30	44.20
30.00	32.40	37.00	41.80	33.90
36.00	19.80	22.60	28.20	18.20
48.00	10.00	12.00	16.20	9.47

Attached to the document are concentration-time tables for the experimental samples. These are reviewed to establish that concentrations reported are within the specified range of quantitation and that all samples received were in fact assayed. The plasma-concentration time data (Table 9) is for one treatment of the above

Table 10. Record of Reassayed Biological Specimens for the Analysis of Cibenzoline in Human Plasma

RECORD OF RE-ASSAYED BIOLOGICAL SAMPLES

TYPE OF SAMPLE	PROTOCOL	INVESTIGATOR
PLASMA	2667A	Hanigan

SUBJECT I.D./hr	ORIGINAL DATA			REASON FOR RE-ASSAY*	RE-ASSAY DATA			COMMENT*	CONC. REPORTED (ng/ml)
	NOTEBOOK REF.	DATE OF ASSAY	CONC. (μg/ml)		NOTEBOOK REF.	DATE OF ASSAY	CONC. (ng/ml)		
001M/48 hr	11679,p.109	7/28/83	3.33	B	11679,p.120	8/29/83	NM	C	NM
002M/36 hr	11679,p.109	7/28/83	4.77	B	11679,p.120	8/29/83	4.30	C	4.30
002M/48 hr	11679,p.109	7/28/83	3.05	B	11679,p.120	8/29/83	NM	C	NM
003M/48 hr	11679,p.109	7/28/83	2.10	B	11679,p.120	8/29/83	NM	C	NM

B = Plasma concentration of 5 ng/ml is less than validated limit of quantitaiton.
C = Sample assayed using new calibration standards with a 2.5 ng/ml limit of quantitation.

bioavailability study for subjects No. 33-36 over the interval of 0-48 hours following the oral administration of 160 mg Cipralan.

Lastly, along with the "In-Vivo" form and described documents is attached a Record of Re-assayed Biological Samples" for the clinical protocol (Table 10). This is a list of all samples reassayed, identified by subject, time and treatment. This list gives notebook reference, dates of assays and concentrations measured for the original and repeat assay. In addition, the reason for reassay is stated and any additional comments are made to support the selection of one concentration over another in the final report. The availability of this reassay data is supportive and required by GLP's.

The assay validation process described for a given assay and/or protocol is repeated for each new assay laboratory and experimental protocol. For the final New Drug Application (NDA) pharmacokinetic submission, analytical summaries of inter-assay precision and quality control results are compiled based on the accumulated validation documentation to describe the performance of an assay throughout the pharmacokinetic drug development process. The inter-assay precision data for cibenzoline (Table 11) was accumulated over the concentration range of 2.5-1000 ng/ml of plasma for seventeen clinical protocols, which represented approximately 200 separate analytical experiments conducted at 3 analytical laboratories. The summary results are reported in terms of mean and ranges of concentrations found and coefficients of variation at each concentration for the studies which demonstrates the overall performance of the assay for the analysis of the clinical samples. The quality control

Table 11. Summary of Inter-Assay Precision Data for Cibenzoline in Human Plasma

Concentration Added (ng/ml)	Mean Concentration Found (ng/ml)	Range of Concentrations Found (ng/ml)	Mean C.V. (%)	Range of C.V. (%)	N^+
2.5	2.6	2.5-2.7	10.6	8.3-15.3	8
5.0	5.0	4.6-5.3	8.3	0.1-13.7	12
10.0	9.8	8.4-10.6	7.0	0.8-13.6	17
20.0	20.3	19.3-21.2	7.4	2.4-14.5	8
25.0	23.6	22.5-24.5	6.8	3.6-9.9	9
50.0	49.3	44.8-52.1	5.7	1.1-10.0	17
100	99.9	94.5-103	4.1	1.6-6.5	17
200	200	191-204	2.6	0.3-4.0	9
250	250	242-254	4.1	2.4-7.2	8
500	505	492-532	3.8	0.4-6.6	15
750	764	738-783	4.5	2.8-8.4	8
1000	1010	951-1080	4.4	0.2-10.5	16

N^+ = Number of clinical protocols

summary data (Table 12) also demonstrated the precision of the assay and in addition provided information on the long term stability of the compound, i.e. the data from quality control pool demonstrated the stability of the compound stored for a period of 15 months at -17°C.

CONCLUSION

A process for validation of the analysis of drugs in biological fluids has been described. The assay validation process is rigorous and may appear extremely time consuming. However, experience has demonstrated that with a uniform, fixed set of validation guidelines

Table 12. Overall Summary of the Analysis of Quality Control Samples for Cibenzoline in Human Plasma

Protocol No.	Dates of Assay	Conc ± S.D. (% C.V.) Pool Identification A	B	C	D	E	F
1	3/82	52.7 ± 1.9 (3.6)	445 ± 20 (4.4)				
2	2-7/82	53.6 ± 2.1 (3.9)	438 ± 14 (3.2)				
3	3-7/82	49.5 ± 1.2 (2.4)	438 ± 14 (3.3)				
4	4-9/82	46.6 ± 3.1 (6.6)	460 ± 20 (4.3)				
5	9-12/82	47.0 ± 2.2 (4.7)		712 ± 29 (4.1)			
6	6-7/83	49.2 ± 1.6 (3.3)		787 ± 37 (4.7)			
7	7-11/83	46.5 ± 2.7 (5.8)		766 ± 28 (3.7)			
8	6-12/83	45.2 ± 1.3 (5.0)		698 ± 42 (6.0)			
9	1-4/83	46.7 ± 2.3 (4.9)		682 ± 33 (4.8)			
10	3-6/83	48.5 ± 1.2 (2.5)		672 ± 32 (4.7)			
11	5-7/84				146 ± 9 (6.2)	421 ± 25 (5.9)	
12	8/84				143 ± 11 (8.0)	426 ± 46 (10.7)	
13	7-12/84				152 ± 7 (4.8)	436 ± 18 (4.2)	
14	8/84				142 ± 11 (7.7)	418 ± 27 (6.3)	156 ± 7 (4.7)
15	7-8/84					440 ± 43 (9.8)	153 ± 11 (7.3)
16	8-9/84					394 ± 19 (4.8)	153 ± 6 (4.1)
17	1-2/85					441 ± 32 (7.2)	160 ± 6 (4.0)

for analytical data treatment, reduction, and reporting that start-up time in an applications laboratory has been greatly minimized. In addition, and certainly more importantly, is the fact that the quality of the analytical data generated has dramatically improved.

REFERENCES

1. M.R. Hackman, T.L. Lee and M.A. Brooks, J. Chromatogr. 273 (1983) 347.