Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, No. 5, pp. 389–400, 1990
Printed in Great Britain

0731-7085/90 $3.00 + 0.00
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Review

Liquid chromatographic methods validation for pharmaceutical products

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Abstract: The current status of validation in LC methods for the analysis of pharmaceuticals has been reviewed with special reference to compatibility testing methods. Validation data were provided in terms of method linearity, accuracy, precision, system suitability, specificity, use of alternate methods, injection order, application of peak height or area measurements and of internal or external standards.

Keywords: *LC; methods validation; review.*

Introduction

The use of LC in pharmaceutical stability and compatibility studies has expanded over the past decade to become the present method of choice. These methods have been adopted by both manufacturing firms and regulatory authorities largely because of their stability indicating properties, their high sensitivity, their automatability and the multiplicity of column chemistry and mobile phase combinations available.

The importance of validation of LC methods in compatibility studies has been seldom discussed in the past despite the fact that these methods must be proven experimentally to function as claimed. The question of how much validation or what kind of validation is necessary in a study supported by LC data has been either unanswered or implicit in a particular journal style. For these reasons and because no general rules have been proposed or adopted as to validation requirements for compatibility studies, the present review was written.

The level or quantity of validation data available is quite variable on a scale from a mere proof of linearity to full fledged studies as would be required by the FDA for methods supporting stability of drug products or analysis of drugs in biological samples. As a minimum it is the duty of the investigator to present sufficient experimental evidence to support the LC method utilized in the particular investigation; to show that the method does what it is supposed to do.

Previous reviews of LC methods validation have highlighted their general aspects [1–3], gone into some detail on particular aspects such as accuracy [4] or ruggedness [5] or presented broad but detailed descriptions of pharmaceutical validations [6–8]. Other reviews or guidelines have been published on methods validation for drugs in biological fluids [9] and in stability testing programmes [10–12] with numerous examples as well as detailed descriptions of each phase of a validation as practised at the time of publication. In the present regulatory atmosphere these studies must be supported by all validation phases previously outlined and more.

Compatibility studies as commonly practised in the past utilizing HPLC have either given reference to the fact that the method was stability indicating [13–15], provided reference and noted retention times of drugs and potential degradation products [16–22] or have included actual forced degradation studies in which drug solutions were subjected to acid, base and thermal stress until some decomposition was observed [23–31].

Selected drug stability studies utilizing HPLC have in general included more information supporting or validating their claims [32–44]. These have included precision, linear-

ity, separation of analogues and degradation products with retention times in addition to forced degradations and actual kinetics measurements.

Survey Method

The actual meaning and utilization of the various phases of a validation are exemplified by a survey of published methods on LC analysis of drug substances and dosage forms. These results are shown in Tables 1–3 for studies on drug substance and solid dosage forms; drug substance, solid and liquid dosage forms and on studies on liquid dosage forms alone including parenterals and aerosols, respectively. The field of validation in LC analysis is biological samples has been excluded altogether.

The columns in each of Tables 1–3 are headed with the titles: Linearity, Accuracy, Precision, System Suitability, Specificity, Alternate Methods, Injection Order, Peak Height or Area, External or Internal Standard, Source and Reference. Linearity (L) indicates that a linearity of detector response–concentration relationship has been established for the drug under consideration covering the expected range of analysis, while an LI has the same meaning for an impurity. Often the impurity linearity measurements are made between 0.1–1.0% of the main component since the FDA normally requires analysis of impurities down to the 0.1% concentration in both drug substance and products. In this same column, MQL indicates that a minimum quantifiable level was measured for the major drug, while MQLI has the same meaning for an impurity. These may or may not be the same as detection limits.

Accuracy

Accuracy is usually established through spiked placebo studies (simulated samples) in which placebo is fortified with drug at various concentrations above and below the target claim. Frequently 0, 80, 100 and 120% or 0, 75, 100 and 125% of claim are used. These samples are then passed through the processing scheme, assayed and the linearity of recovery is calculated with appropriate statistical analysis shown in the next column. The subscripts SP_1, SP_2 . . ., indicate the number of concentrations at which drug was added to placebo, not including the blank (0%) if one was used. Under certain circumstances use of the spiked placebo method is impossible such as in academic settings or in government labs, which cannot obtain authentic placebo and its exact composition is unknown. Here the standard addition method (SA) should be used to verify accuracy by beginning with a sample and then adding known amounts of standard to it in order to derive a linearity expression. This method is also commonly practised in impurity analysis for drug substance in which various levels of impurity are added to the lot of bulk drug showing lowest impurity levels (SP_I). Linearity of recovery of degradation products likewise can be calculated following their addition to placebo for drug products (LR_I). Recovery studies performed using different columns or on different days are designated with these respective subscripts. Drug substance recovery (DSR) studies have been performed which do not relate to method accuracy but only to reproducibility of standard preparation.

Precision

The precision column includes tests for precision of the system (PS) which is measured by replicate analysis of a single standard solution, ordinarily run before initiation of sample analysis as part of a system suitability test. This precision measurement should be carried out on each day a particular analysis is performed giving rise to the expression of results for different days (PS_d). Method precision (PM) is shown by replicate analysis of a pooled sample such as the thoroughly mixed contents from 20 capsules, 20 finely ground tablets or five ampules. Each measured aliquot is carried through the entire sample preparation scheme and assayed. If this measurement was done on more than 1 day it is designated (PM_d) and if it was done using more than one column it is designated ($PM_{columns}$). Precision of recovery (PR) indicates that multiple measurements have been made on placebos spiked at one concentration. Precision of linearity of recovery (PLR) is the measure derived from the linearity of recovery study in which percents recovered at each concentration, possibly in replicate, are analysed to give the RSD. Similarly, precision of linearity of recovery of impurities (PLI) has the same meaning for linearity of recovery of impurities or degradation products added to a drug

substance or to a placebo for a drug product versus a known impurity standard, while PRI indicates that a precision measurement was obtained on recovery of an impurity at one concentration. If a precision determination was made on the minimum quantifiable concentration, this is denoted by PMQL. As part of a ruggedness test, the method precision is determined by assaying the same set of samples in different labs giving PM_r. Method ruggedness is also indicated by results from tests in which standard mixtures are chromatographed using mobile phase variations of 10–20% (organic/aqueous) and by use of one mobile phase with three to five columns of different age for analysis of a standard mixture. A final precision measure that has been determined is a method precision in which different lots of bulk drug are assayed giving PM_L.

System Suitability

System suitability tests are included in the following column which include resolution factor (RF), precision of standard analysis (system precision, PS) or precision of impurity analysis (PI) and can include such measures as tailing factor (TF) or standard linearity (L). Other parameters measured under system suitability can include capacity factor (k'), retention time (t), relative retention (α), number of theoretical plates (N) or peak symmetry (s). These terms have been adequately described in previous reviews and in the USP [45].

Specifications are usually set in both precision and accuracy results for validations ($\pm$1–2%) and for each of the parameters measured in the system suitability test. For example a resolution factor of >2.0 between the peaks for compounds **A** and **B** and a system precision of <1.5% would show how a system performed on a particular day.

Specificity

Specificity studies including the subcategory selectivity, which are not often distinguished in print [6], are shown in the next column. Selectivity implies that the method separates potential process impurities (I), degradation products (D) and structural analogues (A). Specificity, as a broader concept, also includes peak homogeneity. This means that a particular peak corresponds to a single chemical entity rather than several different molecules whether structural, geometrical or configurational isomers or unrelated compounds with overlapping retentions. This property can be indicated by diode-array detection in which spectra taken at various times while a peak is eluting are compared with standard spectra known to be due to a single entity. A second means of showing peak homogeneity is to collect the fraction as the peak elutes and run the sample in an alternate chromatographic system such as TLC or a different mode of LC. Alternatively a non-chromatographic stability indicating method such as capillary electrophoresis or certain electrochemical methods may verify that a collected peak and a standard substance are the same. When a method is shown to be specific for a particular compound, this implies that the method is stability indicating. Further proof of this implication comes from stress studies in which drug product and/or drug substance are degraded chemically (acid, base, oxygen, air), thermally and photochemically. These forced degradations (FD) give rise to reaction products which can be separated from the parent compound and quantified. If this is done as part of a stability study, degradation kinetics (DK) can be established. A stressed placebo study can be included as well to show that no products resulting from possible excipient decomposition will interfere with measurement of components of interest.

Alternate Methods

The use of alternate methods to further substantiate the results of newly developed LC methods is often useful. This is indicated in the next column. Alternate methods have often been previous methods such as non-stability indicating spectrophotometric or titrimetric routines, results of which are listed in tables to show that the new method works at least as well as the old one did.

Injection Order

The following column titled Injection Order shows whether or not any particular order of injection of samples and standards was specified and the number of replicate injections required. Although this order is often only implied, the exact order of standard and sample injections and number of each should be clearly specified. In addition, the method of

Table 1
HPLC methods validation on tablet and capsule dosage forms and drug substance

Linearity	Accuracy	Precision	System suitability	Specificity	Alt. methods	Inj. order	Peak height area	Standard	Source	Reference
				(A)						
L		PS	RF					I	A	47
L								I	A	48
L		PS						I	A	49
L, LI	SP_1	PM		D			H		I	50
				DK					G	51
MQL	SP_1	PM		A	X		A	I	I	52
LI, MQLI	SP_1	PM, PS			X				G	53
				DK	X	B_2	H		G	54
L		PM, PS		D	X		A	I	G	55
LI, MQLI	SP_I			DK		R			G	56
	DSR	PS					H		A	57
				(B)						
LI, L			PS, RF			T	A	E	G	58
L							A		G	59
		PM				R	A	I	I	60
MQLI		PM_d		D		R			I	61
L, MQL	SP_3	PM, PLR		A, D, I		B	H, A	E	I	62
L	SP_1	PS, PM		FD, A			H		I	63
	SP_1	PR	RF, PS, L					I	G	64
MQL	SP_1	PM, PM_d					H		A	65
L		PS	TF				response		G	66
				(C)						
L, MQL			k', S	D					I	67
LI, MQLI	SA, SP_4	PLI, PM, PL		D, A			A		I	68
L, MQLI	SP_6, SA	PS, PM_r		D, DK				E	I	69
	SA	PM		DK	X				I	70
MQLI, L		PM		DK		D	A	I	A	71
L	SP_6, DSR	PM		D			A		G	72
L	SP_1	PS, PM				T	A	I	A	73
L	SP_5	PR, PS, PM		A			A	I	A	74
L, LI, MQLI		PM_r, PM, PS	RF, PI	D	X		H, A	I	G	75
L, LI, MQLI	SP_5, LR_I, SP_I	PM, PRI, PS, PR	RF	DK	X		A		I	76
L	SP_3, SP_I	PR, PRI		FD, D		B	A		I	77
L, MQL, LI	SP_7	PRI, PR		D, A, FD				I	I	78
	SP_1	PS					H	I	A	79

(D)

L	SP_1	PS		FD, DK			H	I	A	80
L		PM_r	*t*, *N*, RF	A, D			A		G	81
MQL, L	SP_3	PR	RF, N, PS, L	FD, D, I		B	A		I	82
MQL, L	SP_3	PR, PM	RF, L, PS, N	D, FD, I		B	A		I	83
L	SP_7	PM	TF, RF						I	84
	SP_1	PM, PS, PLR	TF			B	response		G	85
LI, L, MQLI		PS, PM_I	RF		X	R		I	G	86
L	SP_5	PM	PS, RF, TF				A		I	87
L, MQL, MQLI	SP_3	PM, PLI	RF, PS	D, FD, DK				E	I	88
MQLI		PM	A, D				A	E	I	89
L		PS, PM	PS, RF		X		H	I	G	90
	SP_1	PM, PR			X	B	H		G	91
L	SP_3	PS, PR, PM_r	PS, RF	FD	X	T	A		I	92
MQL, L	SP						H		A	93

Key: L, linearity; LI, linearity of impurities; MQL, minimum quantifiable level; MQLI, minimum quantifiable level of impurity; SP_1, spiked placebo at 1 level; SP_2, spiked placebo at 2 levels; SA, standard addition; LR_I, linearity of recovery of impurity; SP_I, spiked placebo with impurity; SP_{column}, spiked placebo studies on different columns; SP_{days}, spiked placebo studies on different days; DSR, drug substance recovery; PS, system precision; PS_d, system precision on different days; PM, method precision; PM_d, method precision on different days; PM_{col}, method precision on different columns; PR, precision of recovery; PLR, precision of linearity of recovery; PLI, precision of linearity of recovery of impurity; PRI, precision of recovery of impurity; PMQL, precision of minimum quantifiable level; PM_r, precision of method in different labs (ruggedness); PM_L, precision of method for different lots of drug; RF, resolution factor; PS, standard precision (system precision); PI, impurity precision; TF, tailing factor; L, linearity; k', capacity factor; t, retention time; α, relative retention time; N, theoretical plates; S, peak symmetry; I, separation of impurities; D, separation of degradation products; A, separation of analogs; FD, forced degradation; DK, degradation kinetics; X, alternate methods used; B_1, bracket single samples with 2 standards; B_2, bracket 2 samples with 2 standards; B_6, bracket 6 samples with 2 standards; D, duplicate analyses; T, triplicate analyses; R, replicate analyses; A, peak area; H, peak height; I, internal standard; E, external standard; A, academic; I, industrial; G, government.

Table 2
HPLC methods validation for solid and liquid dosage forms and drug substance

Linearity	Accuracy	Precision	System suitability	Specificity	Alt. methods	Inj.order	Peak height area	Standard	Source	Reference
				(A)						
	SP_6	PM, PM_d, PM_r					A	I	I	94
L		PM			X	T		I	A	95
MQLI				D			H		A	96
	SP_5	PM, PM_L		D, A			H	I	I	97
L		PM							G	98
		PM		FD			H	I	A	99
	SP_1	PS, PM_r					H		A	100
L	SP_3	PM, PR, PM_r	*N*, *t*	FD, D, DK	X	B_6	H	I	I	101
L, LI	SP_1	PM, PR		D			H	I	A	102
L	SA	PM	RF, P, TF	A, D, I			A		G	103
L	SP_2	PR, PM_r	RF, N, PS, L	FD			H		I	104
	SP_2			FD			H	I	A	105
L, MQL	SP_3	PMQL, PM, PR	RF, PS				A	I	I	106
L	SP_1	PS, PR, PM		D				E	I	107
				(B)						
MQLI, L, LI, MQL		PM, PLI		FD, D					I	108
L	SP_5	PM, PR, PM_d		A, D			A	I	I	109
L, LI, MQLI	SA	PS	RF, PS				H	I	G	110
	SP_1	PR, PM		FD			A		A	111
	SP_7	PR, PM_d, $PM_{columns}$					A–H	I	I	112
				D					I	113
									I	114
L, MQL	SP_5	PM		FD	X		A	I	A	115

See Table 1 for key to symbols.

Table 3
HPLC methods validation for liquid dosage forms

Linearity	Accuracy	Precision	System suitability	Specificity	Alt. methods	Inj. order	Peak height area	Standard	Source	Reference
				(A)						
	SP_{13}	PM_d, PM		D				I, E	I	116
L	SP_1	PS, PM		FD			A	I	I	117
L		PS, PM					A		G	118
L, LI	SA	PL, PLI	PS, RF	D	X		A	E	G	119
	SP_3	PM, PS				B_2			I	120
L, MQL	SP_3	PLR, PI		A, D	X			E	I	121
	SP_3	PLR		A		B	A	E	I	122
MQL	SP_3	PLR		A		B	H, A	E	I	123
L, LI	SP_3, SP_I	PI, PM		D, FD		B	A		I	124
	SP_3		RF, PS, L	D, FD			A	E	I	125
L	SP_1	PM, PR		D, FD			A	I	A	126
	SP_3	PS, PS_d, PLR	RF, L, PS	A, D, FD	X		H, A		I	127
				(B)						
L	SA	PM						I	G	128
	$SP_{3columns}$, SP_3, SP_{3days}	PR	RF, PS	FD			H, A		I	129
	SP_3	PLR, PS, PS_d	PS, RF, L, TF	FD			H, A	E	I	130

See Table 1 for key to symbols.

calculation should be given, e.g. bracket one sample with two standards, use one standard for the next five samples, use a linear relation between two standards for the next three samples, etc. The symbols used to specify injection order and number of injections are: bracketed (alternate) single samples with standards (B), two samples between two standards (B_2), six samples between two standards (B_6), duplicate injections (D), triplicate injections (T) and replicate injections of an unspecified number (R). While the effect of standard and sample injection order has seldom been discussed previously [5, 46], the importance of number of injections with respect to precision measurements and acceptance range has been documented [10].

Additional considerations

The following column shows whether assay calculations were made based on peak area (*A*) or height (*H*) for each study included. The merits of each measurement for particular applications have been adequately discussed elsewhere.

The use of an internal standard is shown in the next column as (I). Alternately use of an external standard is symbolized by (E). Where neither was specified it is assumed to be external and left blank. Generally internal standards should be unnecessary in drug product or substance stability assays unless dictated by extraction difficulties, as occurs in biological samples, or longstanding corporate policy.

The second last column gives the source of the investigation, either academic (A), industrial (I) or governmental (G).

Interpretation

Since each table was arranged chronologically, any trend in published information on methods validation should be apparent. The only one that is immediately obvious is in Table 1 on solids and drug substances that the information supplied on system suitability testing has increased over the 10 years of this survey. This has probably been in response to regulatory and compendial requirements. The summary of data presented in Tables 1–3 is shown in Table 4 where it can be seen that most (72%) of the studies on solids provided some sort of linearity data, either for the drug in question or an impurity. Fewer (40%) gave detection limits for the compound of interest or an impurity in the solid dosage form and drug substance investigations. Methods accuracy was supported in 57–87% of the studies with the liquids and parenterals showing the highest results, whereas precision of some kind was discussed in the vast majority of papers. Specificity as either forced degradation studies or separation of degradation products or structural analogues were represented in at least half of the papers, some showing data for both, while alternate methods were discussed in far fewer studies. The order of injection and number of standards and samples was most often not described and both peak height and area measurements were reported. Internal standards are still often used with no relationship to whether the study was conducted in an academic, industrial or governmental laboratory.

The recent FDA guideline on submission of samples and analytical data for methods validation has suggested that demonstrations of accuracy, precision and linearity (80–120% theoretical), methods specificity and detection limits for degradation products and their structures be included in drug substance validations [131]. The same information should be supplied for dosage form methods plus recovery studies from sample matrix, evidence for lack of interference from stressed or unstressed placebo, precision measurements between labs, between analysis and between columns and information on drug degradation with LC separations of those products.

The USP also has set forth instructions for validation of proposed methods [132]. Included were definitions and determinations of precision, accuracy, limit of detection, limit of quantitation and selectivity. Linearity and ruggedness were discussed as was a categorization of assay types with different validation requirements for each. Category I dealt with major components in bulk drug substance or the active in drug products while Category II concerned degradation products and impurities. Category III was for dissolution methods.

Conclusions

With these directives on drug substance and product validation requirements and the survey of contemporary validation procedures, it is obvious the extent of method validation in

Table 4
Validation data summary

	Linearity	MQL	SA	SP_1	SP_{2-3}	$SP_{>3}$	*Precision*	System *suitability*	Specificity *FD*	*D or A*	Alt. *methods*	Inj. *order*	Peak *height*	*area*	Internal standard	I	G	A	Total
(1) Solids and drug substance	34	19	2	11	6	8	39	18	8	18	13	15	12	20	16	20	16	11	47
(2) Solids, drug substance and liquids	12	5	2	4	4	5	18	5	7	9	3	2	10	7	12	11	3	8	22
(3) Liquids	7	2	2	2	8	1	14	5	7	9	3	3	4	11	4	11	3	1	15

support of compatibility studies should be expanded. Minimum requirements should include linearity, method precision, accuracy and specificity studies. The linearity range should extend from at least one-half to two times the nominal assay concentration while method precision can easily be demonstrated by a six-fold replicate analysis of a pooled sample. Accuracy is best established through analysis of simulated samples (spiked placebos) although in hospital and academic labs the standard-addition method may adequately provide this information. Method specificity in the broader sense should be proven through either forced degradation studies or separation of potential degradation products from the parent compound. In compatibility studies involving more than one drug product the possible multiple degradation compounds must be adequately separated from each active assayed. Typical compatibility studies should obviously not require the degree of validation necessary to support drug substance and drug product stability and release assay procedures for INDs and NDAs since the main objective of the former studies is to show compatibility or incompatibility between commercial or prototype admixed components. While the clinical importance of these studies should not be underestimated the means necessary to prove their results should be those available to the academic or clinical chromatographer. These facilities may not include diode-array detectors and sophisticated fraction collectors making studies to prove chromatographic peak homogeneity more difficult.

The application of findings in the present review to submissions involving LC supported studies other than compatibility to regulatory agencies and for journal publication is not excluded and will provide some useful information especially by means of comparison of new submissions to those from other sources. This will be especially useful in the present regulatory environment of increased attention to validation portions of submitted methods. The major intent of this review, however, was to make these results available to investigators testing product compatibilities and to suggest that results from these recommended studies should be included in those publications dealing with compatibilities.

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[Received for review 12 September 1989; revised version received 4 December 1989; final version received 4 May 1990]