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Validation Process of HPLC Assay Methods of Drugs in Biological Samples

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생체시료내 약물의 HPLC 분석법에 대한 유효성 검토방법

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An HPLC assay method of a drug to be applied to the pharmacokinetic studies of the drug should be completely validated. The validation process for an HPLC assay method in a biological sample was discussed using the data obtained from the development of HPLC method for the simultaneous quantitation of verapamil and norverapamil in human serum. The validation criteria included were specificity, linearity, accuracy, precision, sensitivity, recovery, drug stability, and ruggedness of an assay method.

Keywords—HPLC, validation, biological sample, verapamil.

To study the pharmacokinetics of a drug, its amount in biological samples should be analyzed with one or more assay methods. The assay methods employed for these studies should be completely validated, because an inaccurate or imprecise assay can result in significant error in the interpretation of obtained data and determination of pharmacokinetic parameters. Validation is the process used to prove that an assay method yields consistently what is expected and required to do with adequate accuracy and precision.

Nowadays, there are many assay methods available for the assay of drugs in biological samples. Nevertheless, high performance liquid chromatographic (HPLC) assay method has become the most popular analytical tool for this purpose for its reproducibility, simplicity, less time-consumption and low-cost. Thus, the validation process presented in this paper will be focussed on HPLC methods for the assay of drugs in biological samples. The same validation process can be applied to the validation of other assay methods with little modification.

In general, the validation of an HPLC assay method of a drug in a biological sample includes evaluation of its specificity, linearity, accuracy, precision, sensitivity and others such as recovery, drug stability and ruggedness. These validation criteria will be discussed separately using the data obtained from the development of the HPLC assay method of verapamil and norverapamil in which the authors were involved.

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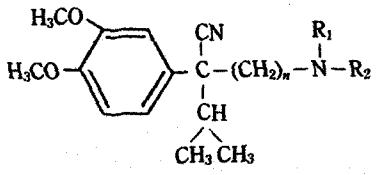


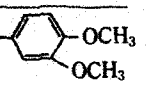
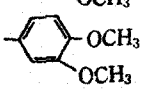
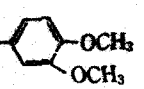
	n	R_1	R_2
Verapamil	3	$-CH_3$	$-(CH_2)_2-$ 
Norverapamil	3	$-H$	$-(CH_2)_2-$ 
D617	3	$-CH_3$	$-H$
D620	3	$-H$	$-H$
Internal Standard (D517)	2	$-CH_3$	$-(CH_2)_2-$ 

Figure 1—Chemical structures of verapamil, its metabolites and internal standard.

HPLC method for the simultaneous quantitation of verapamil and norverapamil in human serum—The simultaneous quantitation of verapamil and norverapamil in human serum using HPLC method was performed as follows. All analytes involved in the assay are shown in Figure 1. Norverapamil was included in the assay, because it is a main metabolite of verapamil in human and has a pharmacological activity similar to verapamil.

To 1.0 *ml* of human serum, aliquots of both verapamil and norverapamil stock solutions were added to simulate 10 different verapamil and norverapamil concentrations ranging from 1 to 500 ng/ml and from 0.5 to 250 ng/ml, respectively. Then, 40 μl of the internal standard solution (D 517 500 ng/ml of pH 4.4 acetate buffer) was added after the spiked serum sample was alkalinized with 100 μl of 1 N NaOH. For the samples containing more than 100 ng/ml of verapamil, 160 μl of the internal standard solution was added. The sample was extracted with 10 ml of cyclohexane : methyl *t*-butyl ether (1:2) by mechanically shaking for 20 minutes and centrifuged at 2000 rpm for 5 minutes. The organic layer was transferred into another glass tube and evaporated under a gentle stream of nitrogen at 30℃ until the volume was reduced to approximately 1 ml. After 200 μl of 0.1 N HCl was added into the tube, the sample was extracted again by shaking for another 20 minutes and centrifuged as explained earlier. Fifty μl of the acidic aqueous phase was injected onto a column using an automatic injector (WISP 710B, Waters).

The column used was Novapak C_{18} (3.9×150 mm, 4 μm particle size, Waters) with a C_{18} precolumn (30 μm pellicular packing, Upchurch Sci.). Mobile phase consisted of 65% methanol and 35% 0.05 M ammonium phosphate dibasic (pH 8.0). Flow rate was 1.0 ml/min and temperature ambient. A fluorescence detector (RF-530, Shimadzu) was used at 280 nm as excitation wavelength and 320 nm as emission wavelength. Peak height ratios between drugs and internal standard were determined with an integrator (SP4270. Spectra Physics).

Three sets of 10 standard solutions of different concentrations were run in a day for intra-day assay validation for a total of 30 analyses and other 3 sets on three different days over one week for inter-day assay validation. The results of all 6 sets of standard solutions were analyzed for the interpretation of linearity, accuracy, precision, recovery and sensitivity of the assay method.

Specificity

The object of specificity test is to ensure that, on chromatograms, the peaks of analytes of interest are free of interference from other components in a sample. The following tests are commonly used to determine the specificity of an assay method.

Selectivity—Selectivity is the ability of an assay method to differenciate a drug in the presence of other interferences such as a) endogenous substances in biological samples, b) metabolites of the drug, c) degradation products of the drug and d) any co-administered drugs and their metabolites or degradation products. Ideally, there should be baseline separation between the drug peaks of interest and all other components for an assay method to be selective.

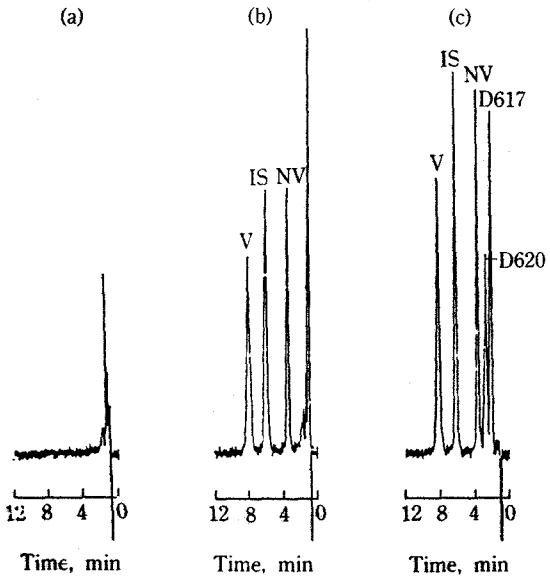


Figure 2—Representative chromatograms of a) blank human serum, b) human serum containing 20 ng/ml verapamil (V), 10 ng/ml norverapamil (NV) and internal standard (IS), c) D617, D620, norverapamil, internal standard and verapamil in mobile phase.

It is not necessary to have baseline separation among components that are not to be quantified. While the interferences by endogenous substances can be determined by assaying a blank biological sample, those by metabolites or degradation products of the drug and/or co-administered drugs can be determined by assaying a bioloical sample which is spiked with these substances. When the metabolites or degradation products are not known, like new drugs, a biological sample obtained from a subject which took the drug should be stored in exaggerated conditions (temperature, pH and light, etc) for a certain period and analyzed to determine whether any possible interference by metabolites or degradation products appears on chromatograms. For the selectivity test of an assay method, it is sometimes necessary to run a blank biological sample more than 30 minutes to confirm that there are no peaks which can be overlapped with the peaks of the next injected sample. As a reference, a blank biological sample should be pooled out of at least three subjects.

Figure 2 shows the representative chromatograms of a) blank human serum and b) human serum spiked with verapamil, norverapamil and internal standard, and c) verapamil, norverapamil, two other N-alkylated metabolites (D617 and D 620) and the internal standard in mobile phase. There was a good baseline separation among verapamil, norverapamil and internal standard. D620 and D617 were overlapped with the peaks of serum extracts, but this was still acceptable since they were not to be quantified. The retention times of verapamil, norverapamil and internal standard were 8.8, 3.7 and 6.7 minutes, respectively.

Homogeneity—In rare cases, a peak may appear on a chromatogram which could be from more than one compound. Homogeneity test can make it sure that the peak is only from the drug of interest, by verifying the integrity of the drug peak by wavelength-ratio comparison to a standard, or by collecting the peak fraction and chromatographing it in another system such as thin layer chromatograph (TLC) or a second HPLC system using different conditions. When wavelength-ratio technique is employed, the spectrum of the drug peak should match that of a reference standard. When the peak fraction is collected and chromatographed in another system, it should appear as a single peak on the HPLC chromatogram or as a single spot with the same retention time as that of the reference standard on TLC.

Linearity

Linearity is the variation in the amount of a drug recovered by an assay method as a function of the amount of the drug actually present in a biological sample. This mainly depends on the ability of the detector to exhibit consistant slope of response to the concentration of the drug.

For linearity test, at least 6 different concentrations of standard solutions covering the expected range of concentrations of a drug in a biological sample for the pharmacokinetics studies of the drug should be processed. To decide the expected concentration range of the drug, the highest concentration in a biological sample at a certain dose

can be expected from literatures or preclinical studies. For the experimental design of verapamil assay, 500 ng/ml verapamil in serum was included as the highest concentration in the experimental range of the drug concentration, because the maximum serum concentration of verapamil was in the range of 150-350 ng/ml at the normal oral dose of 160 mg in man. And the serum concentration of norverapamil at this dose was close to that of verapamil. However, unlike the highest concentration, the lowest concentration of the drug in the biological sample is not set up prior to the assay development, since the more complete pharmacokinetic aspects of the drug can be obtained with more data at the lower concentration using an assay method. Instead, the lowest concentration which can be assayed within acceptable precision and accuracy is determined as the results of the validation of the assay method.

All calibration data obtained from the assay method should be plotted on Cartesian paper and analyzed by linear regression to get calibration lines. Based on each derived calibration line, linearity should be reported with respect to correlation coefficient (r) of the calibration line, slope, intercept as well as their standard deviations. Usually, correlation coefficient should be greater than 0.98 for a new HPLC method of a drug in a biological sample. Any deviation from linearity at any concentration indicates that the assay method does not work properly for the drug at that concentration in the sample. In such case, the assay method in question must be either modified or revalidated to be used for such drug concentration.

The concentration range or the calibration range may be different depending on the drug analyzed, and the purpose of future works with the developed assay method. It can be one order of magnitude, for example 10-100 μg/ml or it can be two orders or more than two orders of magnitude. For data in the range of one order of magnitude, a linear regression analysis without any weight usually suffices the calculation of the calibration line and gives excellent fit of the data. When, however, the range of drug concentration

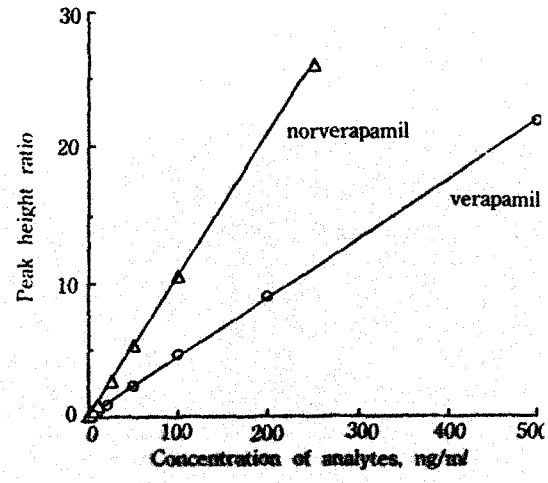


Figure 3—Typical calibration lines obtained by the assay of human serum spiked with verapamil, norverapamil and internal standard (Day III).

to be validated exceeds one order of magnitude, a weighted regression analysis gives better fits due to a more equitable distribution of error on a relative basis instead of an absolute basis.

Figure 3 shows the typical calibration lines for verapamil and norverapamil assay in human serum over the entire concentration range determined with the weighted linear regression analysis using the weight of 1/(peak height ratio). Correlation coefficients of all calibration lines for verapamil and norverapamil assay in human serum were >0.999 and >0.998, respectively.

Precision

Precision is the reproducibility of measurements by an assay method for a drug in a biological sample or the scatter of a number of independently replicated measurements about its average value (not necessarily the true value). This test includes both system precision due to instruments variability and method precision due to the variability in sample preparation other than instruments. It is usually measured and reported in terms of % CV (coefficient of variation) or RSD (relative standard deviation) around the average value for a group of measurements.

The precision of an assay method should be

Table I—*Intra-day precision and accuracy of verapamil assay in human serum*

Std. conc (ng/ml)	Conc found (ng/ml)	% deviation	% CV
1	0.9443[a]	−5.57	20.87
	0.9845[b]	−1.55	
	1.359[c]	35.88	
3	2.905	−3.16	6.49
	2.842	−5.27	
	3.203	6.78	
5	4.999	−0.01	3.43
	4.673	−6.54	
	4.789	−4.23	
10	10.10	1.03	2.05
	9.772	−2.28	
	9.732	−2.68	
20	20.24	1.19	1.54
	19.65	−1.77	
	19.80	−0.98	
50	47.01	−5.99	8.45
	55.41	10.82	
	49.72	−0.56	
100	105.0	5.02	1.19
	105.1	5.11	
	102.9	2.85	
200	210.1	5.06	2.98
	211.0	5.52	
	199.9	−0.04	
500	488.8	−2.24	0.20
	481.6	−3.68	
	498.1	−0.38	
average			3.29

[a]1st analysis; ratio=0.04154×conc+0.00632
[b]2nd analysis; ratio=0.04314×conc+0.00840
[c]3rd analysis; ratio=0.04289×conc−0.00139
*All calibration lines were calculated between 3 and 500 ng/ml. The data at 1 ng/ml were shown after recalibrated between 1 and 500 ng/ml.
**weight=1/peak height ratio

Table II—*Intra-day precision and accuracy of norverapanil assay in human serum*

Std. conc (ng/ml)	Conc found (ng/ml)	% deviation	% CV
0.5	0.5644[a]	12.87	13.77
	0.5068[b]	1.35	
	0.4274[c]	−14.52	
1.5	1.539	2.63	2.92
	1.510	0.69	
	1.453	−3.15	
2.5	2.468	−1.29	3.98
	2.432	−2.74	
	2.288	−8.47	
5	4.950	−1.01	3.42
	4.623	−7.55	
	4.804	−3.92	
10	9.913	−0.87	2.16
	10.31	3.10	
	9.959	−0.41	
25	27.35	9.40	2.70
	25.96	3.84	
	26.99	7.97	
50	45.96	−8.09	6.96
	52.01	4.02	
	51.95	3.90	
100	99.53	−0.47	5.24
	101.6	1.58	
	109.8	9.82	
250	252.9	1.17	3.04
	242.8	−1.67	
	238.4	−4.63	
average			3.80

[a]1st analysis; ratio=0.10879×conc−0.01848
[b]2nd analysis; ratio=0.11182×conc−0.00789
[c]3rd analysis; ratio=0.10533×conc+0.03198
*All calibration lines were calculated between 1.5 and 250 ng/ml. The data at 0.5 ng/ml were shown after recalibrated between 0.5 and 250 ng/ml.
**weight=1/peak height ratio

evaluated with respect to the reproducibility within a day (intra-day precision) and among different days (inter-day precision). For intra-day precision, at least three sets of standard solutions of different concentrations covering the entire range of calibration should be assayed within a day. Separately, for inter-day precision, one set of the same standard solutions should be assayed one day and this should be repeated on at least two other days. The days on which the assays are performed for inter-day precision test should be randomly selected for a long period. However, one week is usually favored for the period of this test, even though this limitation does not follow the

Table III—*Inter-day precision and accuracy of verapamil assay in human serum*

Std. conc (ng/ml)	Conc found (ng/ml)	% deviation	% CV
1	1.314[a]	31.44	19.68
	0.9118[b]	−8.82	
	1.314[c]	31.38	
3	3.292	9.74	7.05
	2.889	−3.71	
	3.250	8.35	
5	4.916	−1.67	5.99
	5.308	6.16	
	4.722	−5.56	
10	10.11	1.10	5.03
	9.289	−7.11	
	10.18	1.82	
20	19.62	−1.86	3.45
	20.10	0.52	
	18.77	−6.13	
50	47.91	−4.19	3.15
	50.78	1.56	
	48.35	−3.30	
100	99.93	−0.07	1.93
	103.7	3.72	
	100.9	0.88	
200	186.7	−6.67	7.62
	202.6	1.29	
	217.5	8.76	
500	517.2	3.34	3.23
	493.7	−1.27	
	486.3	−2.74	
average			4.68

[a]Day II analysis; ratio=0.04371×conc−0.02889
[b]Day III analysis; ratio=0.04420×conc+0.00638
[c]Day IV analysis; ratio=0.04213×conc−0.00894
*All calibration lines were calculated between 3 and 500 ng/ml. The data at 1 ng/ml were shown after recalibrated between 1 and 500 ng/ml.
**weight=1/peak height ratio

Table IV—*Inter-day precision and accuracy of norverapamil assay in human serum*

Std. conc (ng/ml)	Conc found (ng/ml)	% deviation	% CV
0.5	0.4820[a]	−3.60	13.94
	0.6385[b]	27.69	
	0.5784[c]	−15.69	
1.5	1.363	−9.10	9.64
	1.645	9.64	
	1.578	5.21	
2.5	2.536	1.45	1.66
	2.620	4.81	
	2.594	3.76	
5	5.325	6.50	5.47
	4.775	−4.55	
	5.037	0.74	
10	9.464	−5.36	3.04
	9.077	−9.23	
	9.634	−3.66	
25	24.09	−3.64	1.15
	24.63	−1.48	
	24.49	−2.06	
50	54.13	8.26	4.46
	51.32	2.64	
	49.56	−0.89	
100	108.7	8.70	7.84
	101.1	1.15	
	92.89	−7.11	
250	239.9	−4.03	3.85
	249.0	−0.41	
	259.1	3.65	
average			4.64

[a]Day II analysis; ratio=0.10147×conc+0.00865
[b]Day III analysis; ratio=0.10454×conc+0.00507
[c]Day IV analysis; ratio=0.11420×conc−0.03622
*All calibration lines were calculated between 1.5 and 250 ng/ml. The data at 0.5 ng/ml were shown after recalibrated between 0.5 and 250 ng/ml.
**weight=1/peak height ratio

assumption of random selection.

After the concentraions are calculated by re-fitting peak response ratios obtained with different standard solutions into a derived regression equation from the set of these standard solutions, % CVs are determined at each concentration of standard solutions from their average value and standard deviation as follows:

$$\% \text{ CV} = \frac{\text{standard deviation}}{\text{average}} \times 100$$

Table I and Table II show the intra-day precision data for verapamil and norverapamil assay in human serum over the concentration range of

1-500 ng/ml for verapamil and 0.5-250 ng/ml for norverapamil, respectively. All % CVs were less than 10% except those at the lowest concentrations (1 ng/ml for verapamil and 0.5 ng/ml for norverapapmil) and average % CVs were 3.3% for verapamil and 3.8% for norverapamil excluding the % CV at the lowest concentrations. The high % CV at lower concentrations is typical, which is due to the imprecision in measurement of peak responses at these concentrations.

Table III and Table IV show the inter-day precision data for verapamil and norverapamil assay in human serum, respectively, over the same concentration range. The average % CVs were 4.7% for verapamil and 4.6% for norverapamil excluding the % CV at the lowest concentrations.

Accuracy

Accuracy is the index of closeness of the drug concentrations determined by the assay method to their true or known concentrations in a biological sample. It is expressed and reported as % deviation. It is the most important parameter in the assay validation of a drug when the assay is used actually for future works.

The accuracy of an assay method can be determined similar to precision test. By re-fitting peak response ratios from standard solutions of known concentrations (or conc added) into a derived regression equation, the calculated concentrations (or conc found) can be obtained. The "conc found" and "conc added" are then used to determine the % deviation at each concentration of standard solutions as follows:

$$\% \text{ deviation} = \frac{\text{conc. found} - \text{conc. added}}{\text{conc. added}} \times 100$$

Generally, % deviation should be less than 10% across the entire calibration range. Although one can get good precision with an assay method, it does not always mean good accuracy, because any systemic error can lead to precise but inaccurate assay results.

Even though correlation coefficient of calibration lines appears to be close to 1 over the concentration range used in a linearity test, % deviation at low concentrations may show very high value when the assay is validated over a wide concentration range. However, in the study of pharmacokinetics of a drug, some important pharmacokinetic parameters, such as elimination rate of a drug or its half-life, are determined at low concentrations of the drug. Therefore, it is essential to give higher weight to these lower concentrations in order to get more reliable pharmacokinetic data from the accurate measurement at low concentrations. Although a weighted regression analysis may decrease the correlation coefficient value compared to that of a unweighted regression analysis, the weighted regression analysis improves the accuracy of an assay method dramatically, especially, at low drug concentrations.

The intra-day accuracy data, expressed as % deviation of verapamil and norverapamil assay in human serum are included in Table I and Table II, respectively. Their inter-day accuracy data are also shown in Table III and Table IV, respectively. Shown in these tables, both intra-day and inter-day accuracy, as % deviation, were <10% for verapamil between 3-500 ng/ml with one slight exception and for norverapamil between 1.5-250 ng/ml.

Sensitivity

Sensitivity is originally defined as the slope of a calibration line. Thus, the larger change in instrumental response by a small change in drug concentration means the higher sensitivity of an assay method. However, in the validation of an HPLC assay method, the following three terms are routinely used to determine and report the sensitivity of the assay method.

Limit of detection—The limit of detection is the lowest concentration or amount of a drug in a biological sample which is detectable reliably and distinguishable on chromatograms from the noise purely due to the instruments used. The limit of detection should be lower than the lowest concentration of the concentration range used in the linearity test.

Quantitative approach for the determination of the limit of detection is to measure the height of noises on chromatograms and determine the drug concentration which shows the peak height equivalent to the average value plus 3 times its standard deviation. However, 2 or 3 times the signal-to-noise (S/N) ratio is commonly employed to determine the limit of detection. That is, a stan dard solution of an analyte is consecutively diluted and analyzed until the peak height of the ana lyte reaches 2 or 3 times the noise height of instruments at the most sensitive instrument setting. In the verapamil and norverapamil assay, the limits of detection were 0.6 ng/ml for verapamil and 0.3 ng/ml for norverapamil based on 2 times S/N ratio method when 50 μl of the diluted standard solutions was injected. While the concentration of a drug injected can be used as the unit for the limit of detection with fixed injection volume as shown in the verapamil and norverapamil assay, it has become more popular to use the amount of a drug injected as the unit to express the limit of detection.

Limit of quantitation—The limit of quantitation is the validated lowest concentration that can be determined with acceptable precision and accuracy. Ten % CV and 10% deviation can be generally used as the acceptable precision and accuracy limits, respectively, as explained previously. However, the following method was also favored in our laboratories. This method did not set any sensitivity limits, such as 2 or 3 times S/N ratio or any specific acceptable % CV at the lowest concentration, but included an appropriate interpretation of the inter-day precision data. As shown in Table II, the inter-day precision experiment for verapamil assay, there was a concentration at which the % CV began to increase suddenly, as the drug concentration was decreased. The % CV at 1 ng/ml was 40.0%, which was much higher than those at other higher concentrations. Therefore, the next concentration (3 ng/ml) was regarded as the limit of quantitation for the verapamil assay. The limit of quantitation for norverapamil was determined as 1.5 ng/ml based on the same idea. In some laboratories, 10 times S/N is also routinely used to determine the limit of quantitation.

Range of quantitation—It is the range of drug concentrations which can be quantitated with acceptable precision and accuracy. The lowest limit of this range is equivalent to the limit of quantitation. The highest limit is the highest concentration used in the assay validation. However, if CV level and % deviation at the highest concentration is not reasonable, next lower concentration is determined as the highest limit, and so on. For verapamil and norverapamil assay in human serum, 3 ng/ml and 1.5 ng/ml were decided as their lowest limits, respectively, which were their limits of quantitation. On the other hand, 500 ng/ml and 250 ng/ml were decided as the highest limit, because the % deviations and the % CVs at these concentrations were not significantly higher than the average values.

Others

Recovery—Recovery is the detector response to pure standard of a drug in mobile phase compared to the response to the drug of equivalent amount added to and recovered for a biological sample. It mainly depends on the extraction procedure of an assay method. The overall recovery over entire concentration range should be determined for all analytes and for the internal standard in biological samples. Although 100% recovery is ideal, recovery greater than 75% could be acceptable if it is reproducible. However, if it is variable and unpredictable, the reason for this should be investigated and eliminated or an alternative approach for the isolation of the analyte should be looked for. It is strongly recommended to look for a better extraction system as possible at the initial stage of assay method developement, because the sensitivity, accuracy and precision are greatly dependent upon the recovery of the analytes.

There should be no statistical difference in recoveries at low and high drug concentrations. At low concentrations, significant drug loss can often occur with hydrophobic drugs which strongly ad-

sorb to glass. Since recovery normally improves at high drug concentrations due to the saturation of adsorption sites, this adsorption could result in a nonlinear calibration line.

The recoveries for verapamil, norverapamil and internal standard were also determined at each concentration of standard solutions over entire calibration range. The average recoveries were 98.6% for verapamil, 93.6% for norverapamil and 98.1% for the internal standard.

Stability of analytes—Analytes must be stable from the sampling time to the end of their assay because their instability can result in low recoveries and consecutively inaccurate and imprecise data during the development and validation process of their assay methods. They can be lost to their containers or decomposed chemically or enzymatically during handling and storage. These potential problems can be avoided by obtaining stability data of the analytes in various conditions such as a wide range of pH, temperature, light, and in the different solvent systems used in the assay.

Sample solutions and standard solutions should be prepared and injected according to the developed assay method and the solutions should then be reinjected after standing for a length of time equivalent to the expected maximum analysis time with the assay method.

Calculation of the peak responses of initial and final injections should agree to within three times the % CV found in precision test. The chromatogram of final injections should not exhibit any peaks absent from initial chromatograms. If automated runs lasting overnight are expected using an automatic injector, solutions should be checked for their stability over a period of from 12 to 15 hours.

Verapamil, norverapamil and internal standard solutions were found to be very stable when they were stored at pH 4-5 in a refrigerator. Therefore, 0.05 M acetate buffer (pH 4.4) was used as the solvent to prepare the standard solutions of the analytes every month.

Ruggedness—A new assay method may lack the ruggedness to show same results with adequate reproducibility by analysts using different instruments in other laboratories. Since any variation in different experimental conditions should not affect the assay results significantly when an assay method is reproduced, analyst-to-analyst, instrument-to-instrument, and lab-to-lab ruggedness should be carried out. This test can be performed by letting an analyst, who is not involved in the development of the assay, follow the protocol of the developed assay method using different instruments in another laboratory and comparing his results with the validated data. However, this test is not always required in the validation process, especially when the assay is developed and used in the same laboratory.

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