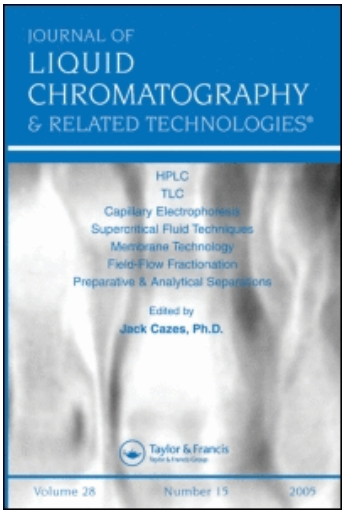

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APPLICATION OF THE ICH GUIDELINES IN VALIDATION OF A CHROMATOGRAPHIC METHOD FOR CCK-4 FRAGMENT OF CHOLECYSTOKININ

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ABSTRACT

This paper describes a simple and rapid procedure for the quantitation of the CCK-4 fragment of cholecystokinin by reversed phase high-performance liquid chromatography (RP-HPLC). The eluent for suitable separation on a C-18 column as stationary phase, was a water/acetonitrile (70/30, v/v) mixture with 0.05 trifluoroacetic acid, pH 2; UV detection was set at 280 nm. The method was developed and validated according to the International Conference on Harmonization (ICH) guidelines. The results obtained in the validation process, indicate

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that the method is specific, linear, accurate (recovery mean = $100.2 \pm 3.0\%$), and reliable (precision = 1.21%). Limit of detection is established for 10.9 μg/mL and limit of quantitation for 33.1 μg/mL. In addition, the usefulness of the method, in order to evaluate the stability of the compound, was demonstrated.

INTRODUCTION

As a result of the increasing interest of peptides and proteins in academic research and pharmaceutical industry, the chromatographic separation of this class of compounds has become one of prime interest. With the development of high-pressure pumping systems providing constant flow-rates, and pressure-stable micro-particulate packing materials, high-performance liquid chromatography (HPLC) has become available to the protein chemist.[1–4] At present, HPLC in reversed-phase (RP-HPLC) separation mode is currently used as a routine technique for separation of peptides and proteins.[5–9] Chromatographic methods are frequently used for the qualitative and quantitative analysis of drug substances, drug products, raw materials, and biological samples throughout all phases of drug development, from research to quality control. However, the analytical method validation is necessary in order to ensure that an analytical methodology is accurate, specific, reproducible, and rugged over the specified range that an analyte will be analyzed. Therefore, the objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. There are several reported guidelines for the practical validation of analytical methods.[10–13]

Among official ones are those issued by the United States Pharmacopoeia (USP), the International Conference on Harmonization (ICH), and the Food and Drug Administration (FDA), which provide a framework for the validation process.[14–16] In a 1987 guideline (*Guideline for Submitting Samples and Analytical Data for Methods Validation*), FDA designated the specifications in the current edition of the USP as those legally recognized when determining compliance with the FDA. For method validation, these specifications are listed in USP chapter ⟨1225⟩.[14] In addition, since the first meeting of the ICH of Technical Requirements for Registration of Pharmaceuticals for Human Use in 1991, several guidelines have reached, or approached, the final stage of the ICH process that will impact the development and validation of HPLC methods.[14,15] The method validation guidelines fall under the quality topics in section Q2, Validation of Analytical Procedures. The harmonized ICH text of Q2A, *Definitions and Terminology*, was finalized (step 4) in October 1994. This guideline identified the validation parameters required for analytical methods. It also discussed the characteristics that must be considered during the validation of

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analytical procedures that are part of the registration process. The harmonized ICH text of Q2B, *Methodology*, was finalized (Step 4) in November 1996. Q2B extended Q2A to include the actual experimental data required, as well as statistical interpretation for the validation of analytical procedures. Both of these guidelines significantly affect people working in the validation area, and users should consult them as they will be incorporated into the next publication of the USP, and federal regulators have already begun to reference these documents. Some of these guidelines are already being implemented by FDA.[13]

In this context, the objectives of this work were: first: the development and validation of an RP-HPLC quantitative method, using the CCK-4 fragment of cholecystokinin as model drug due to their potential diagnosis and therapy uses in nuclear medicine, determining its specificity, linearity, accuracy, precision, range, detection, and quantitation limits and robustness, according to the ICH guidelines.[14,15] The second objective, is to test the usefulness of this method to evaluate the stability under the conditions described by Rossin et al.[17]

EXPERIMENTAL

Chemicals and Reagents

CCK-4 was purchased from Sigma (Sigma-Aldrich, Milan, Italy, Batch: 28H0783). Trifluoroacetic acid (Sigma-Aldrich, Milan, Italy) and acetonitrile (Romil Ltd., Cambridge, UK) were HPLC grade. De-ionized water was purified in a MilliQ plus system from Milllipore, prior to use.

Apparatus and Chromatographic Conditions

The HPLC system consisted of a LKB model 2249 programmable gradient pump (Pharmacia-LKB, Stockholm, Sweden), a Rheodyne model 7125 injector (Rheodyne, Rohnet Park, CA, USA), a Nova Pack C-18 column (150×3.9 mm, 60Å, 4 μm particle size, Waters) equipped with a Hamilton PRP-1 precolumn (Hamilton, Reno, NV, USA). The column effluent was monitored at 280 nm with a LKB model 2140 rapid detector. The data collection and analysis were performed using the Nelson Analytical Chromatography program (Pharmacia-LKB, Stockholm, Sweden).

The mobile phase was an acetonitrile–water (30 : 70, v/v) mixture with 0.05% trifluoroacetic acid (pH = 2), at a flow rate of 1.0 mL/min for 5 min, and the injection volume was 25 μL. The mobile phase was filtered with 0.45 μm (pore size) filters (Millipore) and degassed.

Preparation of Stock and Standard Solutions

CCK-4 (1 mg) was accurately weighed and dissolved in mobile phase to a volume of 10 mL in a volumetric flask. This solution was diluted with the mobile phase as needed to prepare different standard solutions over a range of concentrations of 40–140 μg/mL and analyzed the same day. This process was performed for five times. Each standard solution was injected into the liquid chromatographic system. The peak area of all the tested concentrations were used to construct a standard calibration curve to test the linearity and regression coefficient ($r2$) of the RP-HPLC method.

Stability Studies

A 10 mg/mL peptide solution in DMSO was transferred to a 5 mL volumetric flask, and the pH was adjusted to 11 with NaOH solution (0.01 M). The mixture was heated and kept at 50°C for 4 hours, then cooled to room temperature. The sample was appropriately diluted with the mobile phase to obtain concentration values within the calibration range, and immediately was injected into the chromatographic system to detect peaks of degradation products.

RESULTS AND DISCUSSION

Table 1 shows the similarities and differences of the method validation parameters between the ICH/FDA–USP. The main difference in the USP and ICH terminology is a question of semantics, with one important exception.

Table 1. Differences and Similarities in USP and ICH Method Validation Parameters

United States Pharmacopoeia	International Conference on Harmonization
Precision	Precision
Accuracy	Accuracy
Limit of detection	Limit of detection
Limit of quantitation	Limit of quantitation
Specificity	Specificity
Linearity and range	Linearity and range
Ruggedness	Robustness
Robustness	System suitability

International Conference on Harmonization treats system suitability as a part of method validation, whereas the USP treats it in a separate chapter.[13] It is expected that the ICH definitions and terminology will eventually be published in the USP, since, at present, this guideline has reached step 5 of the ICH process, and the FDA has begun to implement it.[13] In this work, the method validation will be presented according to the ICH guideline. However, it is possible to distinguish between two types of parameters, those related with the method development and optimization (specificity, system suitability, and robustness), and on the other side, the method validation parameters (linearity and range, detection and quantitation limits, precision and accuracy). For this reason, we have divided the RP-HPLC method validation procedure in two parts: first, development and optimization, and second, validation parameters.

Once data are generated, statistically valid approaches should be used to evaluate the data and make decisions, thus, removing some of the subjectivity of method validation.

Method Development and Optimization

Specificity

Successive individual solutions of CCK-4 peptide were injected in the HPLC system, and no interferences were shown. Figure 1(A) shows a typical chromatogram of a standard solution of the studied peptide ($C = 100\,\mu g/mL$); unique peak with a retention time of 2.40 ± 0.08 min was detected. Process-related impurities, either as solvents, buffers or as other components, did not interfere with the peptide.

To demonstrate that this analytical procedure is specific for this particular analyte, the following strategy was used: a CCK-4 sample was stored under relevant stress conditions; temperature of 70°C for 1 hour at pH = 11 in order to provoke the CCK-4 degradation. In this case, the peak of the decomposition product was separate from the peak of CCK-4 [see Fig. 1(B)] with an excellent resolution ($R_s = 4.0$) and high selectivity ($\alpha = 1.71$). Identification of the degradation product was made by fast atom bombardment mass spectrometry (FAB-MS) analysis, corresponding to the oxidized CCK-4 form (data not shown).

System Suitability

According to ICH, the system suitability is a part of method validation. Parameters such as plate count, tailing factor, resolution between the peaks of

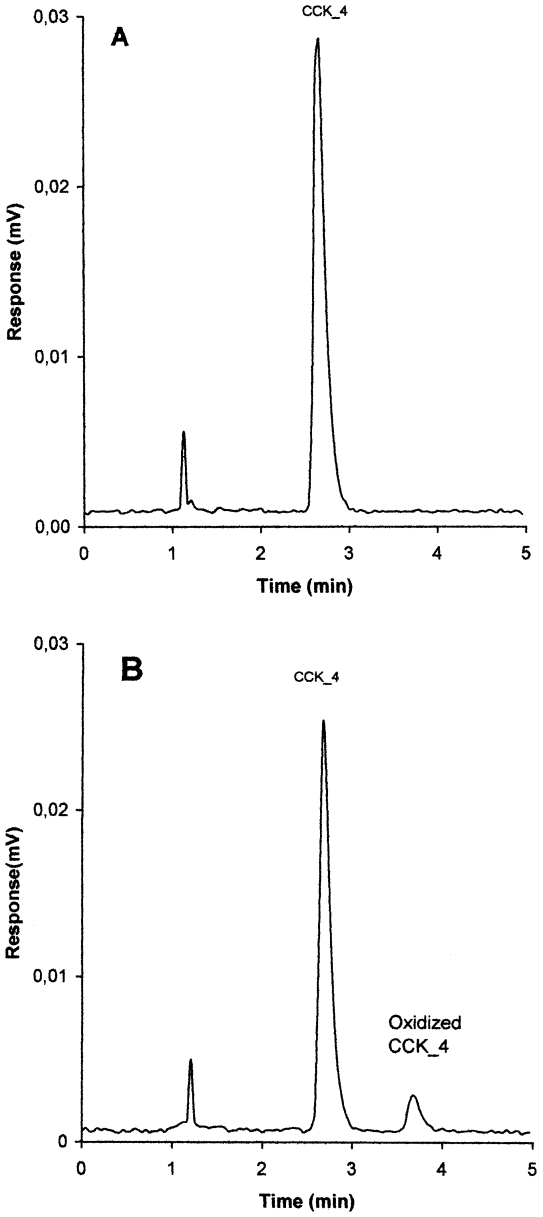

Figure 1. Reversed Phase High-Performance Liquid Chromatography separation of CCK-4 peptide on a Nova Pack C-18 (3.9×150 mm) column with UV-Vis detection at 280 nm. (A) Pure peptide. (B) Sample subjected to degradation ($T = 50^{\circ}$C, pH 11, 1 h).

interest, and reproducibility (%RSD retention time and area for six repetitions) were determined during the study (see Table 2).

With respect to the capacity factor (K'), it is outside of the specified limits by ICH guideline, although in this case, the K' value is not important for controlling separations, since the peak of interest is well resolved from solvent peaks. Reproducibility, expressed as %RSD for peak area for six repetitions, was 1.21%, which is slightly higher than the recommended value ($\leq 1\%$). Reproducibility must reflect the variations due to the instruments and not the analyst. In this case, a manual injector was used. The ICH guidelines do not distinguish between automatic and manual injector. The rest of the studied parameters were inside the recommended limits for the ICH guidelines.

Robustness

The evaluation of robustness should be considered during the development phase, depending on the type of procedure under study. It should show the reliability of an analysis with respect to small deliberate variations in method parameters. For this, the method parameters, such as the flow-rate (1.0 ± 0.05 mL), pH (2.0 ± 0.2), mobile phase composition (30 ± 1 for acetonitrile, 70 ± 1 for water, v/v), and column performance over time, were determined during the validation period to confirm the method's robustness. In order to evaluate the effect of organic % of mobile phase, the capacity factor variations was studied. Small changes in the organic % ($\pm 1\%$) of the mobile phase did not affect the capacity factor and relative retention time, since these parameters were inside the established limits.

Table 2. Reversed Phase High-Performance Liquid Chromatographic System Suitability Parameters for CCK-4 Peptide

Parameter	Obtained Values	Recommended Limits
Relative retention (min)	2.42 ± 0.08	(see note[c])
Capacity factor (K')	1.28	$K' > 2$
Resolution (R_s)	N.D.	$R_s > 2$
Repeatability[a]	1.21%	RSD $\leq 1\%$ ($n \geq 5$)
Tailing factor (T)	1.1	$T < 2$
Theoretical plates (N)[b]	18,500	$N > 2{,}000$

[a]Expressed as %RSD for six determinations.
[b]Theoretical plates calculated using the 5 sigma method.
[c]Not essential as long as the resolution is stated.
N.D.: Not determined.

The stability of samples during the method validation and analysis of unknown sample was determined to ensure that the validity of the analytical procedure is maintained whenever used. A standard solution stored at 2–8°C, was used to evaluate the sample stability. Each work day, a standard solution was analyzed and the peak area was calculated, relative standard deviation (RSD) being less than 2.9% ($n = 6$).

Method Validation Parameters

Linearity and Range

To validate the analytical method, six standard solutions were prepared at concentrations of 40–140 μg/mL. Each sample was analyzed five times. To quantify CCK-4, we used the peak area. The analysis of variance (ANOVA) of the linear regression "peak area vs. prepared concentration of peptide" confirmed the linearity of the method through rejection of the null hypothesis of deviation of the linearity for a significance level of 0.05 ($\alpha = 0.05$); the RSD was 3.74%. The equation of the regression line was:

$$\text{Peak area} = (-352.2 \pm 1838.3) + (1064.2 \pm 19.1)C;\ r = 0.995,\ (n = 30)$$

and the root mean square error (S_{yx}) was 3572.7.

Limit of Detection and Quantitation

Limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated, whereas, the limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The calculation method is based on the standard deviation (SD) of the response and the slope of the calibration curve (S) according to the following formulas: LOD = 3.3 (SD/S) and LOQ = 10 (SD/S), where the SD of the response is determined on the residuals standard deviation of the regression line. Results show LOD = 10.9 μg/mL and LOQ = 33.1 μg/mL, respectively.

Accuracy

To document accuracy, the ICH guideline methodology recommends collecting data from a minimum of nine determinations over a minimum of three concentrations levels covering the specified range (for example, three

concentrations, three replicates each). The data should be reported as the % recovery of the known added amount (see Table 3). The average percentage recoveries were found to be 100.2% with an RSD of 3.00% ($n = 9$).

Precision

Expressed as repeatability the HPLC system, precision was assessed using a minimum of six determinations of a CCK-4 sample at 100% of the test concentration. The repeatability was $<1.21\%$ for the peak area ($n = 6$).

On the other hand, the intermediate precision expressed as the variability between days, was evaluated jointly by one-way ANOVA. A CCK-4 solution sample, at three different concentrations, was analyzed in triplicate on different days, under the same conditions (same analyst, apparatus, identical reagents, and short interval of time). Table 3 summarizes the results for each peptide concentration by day and provides their overall means and coefficients of variation (CV). The CV obtained on the same day (intra-assay precision) was $<3.28\%$; the results of one-way ANOVA showed that the inter-assay differences were not significant ($P < 0.05$). The inter-assay precision was better than 3.13%.

In contrast, the precision expressed as the reproducibility, refers to the results of collaborative studies between laboratories, which should be considered in case of standardization of any analytical procedure, for instance, for inclusion of procedures in Pharmacopoeias. Documentation in support of inter-laboratory precision studies includes the RSD of the different parameters used to evaluate the precision: retention time, resolution, capacity factor, and peak area (see Fig. 2). The three first parameters are directly related to the method development and optimization, whereas, the last one is related to the method validation parameters. Results indicate that the precision was higher for laboratory No. 2 in all parameters studied. An explanation of this observation could be due to the characteristics of the HPLC system used among other factors.

Table 3. Summary of Intra-Day and Inter-Day Precision Data for RP-HPLC Method of CCK-4 Peptide

Concentration (μg/mL)	Day 1	Day 2	Day 3	Inter-Day
40	103.6 (1.86)	97.3 (1.06)	102.4 (0.61)	101.1 (3.13)
100	98.3 (1.93)	103.1 (0.65)	99.4 (0.78)	100.3 (2.10)
140	101.8 (0.82)	99.5 (0.78)	101.6 (0.75)	100.9 (1.19)
Intra-Day	100.6 (3.28)	101.2 (1.80)	100.5 (1.84)	—

Precision was expressed as average percentage recoveries and CV (in parenthesis). Unless otherwise indicated, the daily means corresponding to triplicate injections.

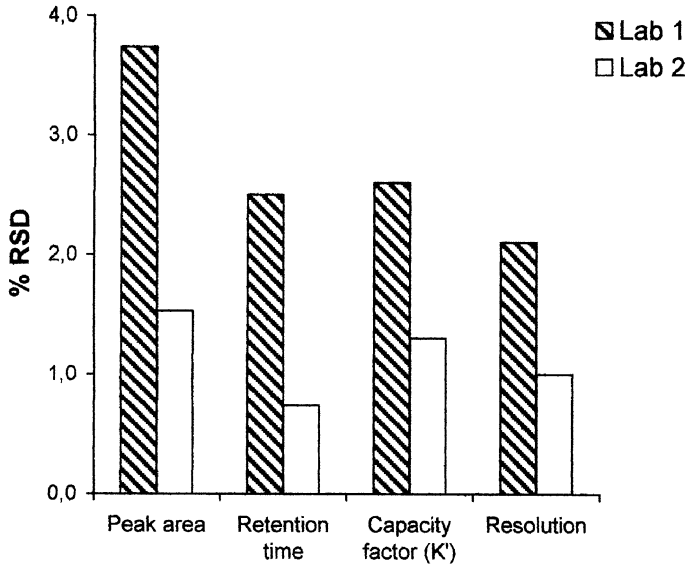

Figure 2. Relative standard deviation corresponding to the different parameters used in the inter-laboratory precision study. In this case, resolution (R_s) between the peak of interest and the peak of process-related impurities was calculated.

CONCLUSIONS

This work describes a simple and rapid methodology for the separation and quantitation of CCK-4 peptide through the use of an isocratic elution mode and a basic HPLC device. The results of the validation procedure, according to the ICH guidelines, show that the method used is specific, precise, reproducible, and robust. The method permitted studying the stability of the CCK-4 peptide under the labeled conditions ($T = 50°C$, pH 11) proposed by Rossin et al.[17] The results indicate, that the product is stable in the conditions described earlier, since the degradation product percentage, identified as oxidized CCK-4 form, was $<1\%$ for 30 min, increasing this percentage up to 7.3% after 6 hours. In addition, the developed method could be used for the simultaneous analysis and quantitation of CCK-4 and CCK-8 fragments of cholecystokinin samples with good resolution ($R_s = 2.0$) and high selectivity ($\alpha = 1.22$), as can be seen in Fig. 3, with an analysis time lower than 5 min.

Finally, in the ICH guidelines, two very different groups of parameters implicated in the validation of chromatographic methods can be observed, where the method development and optimization parameters should be the most

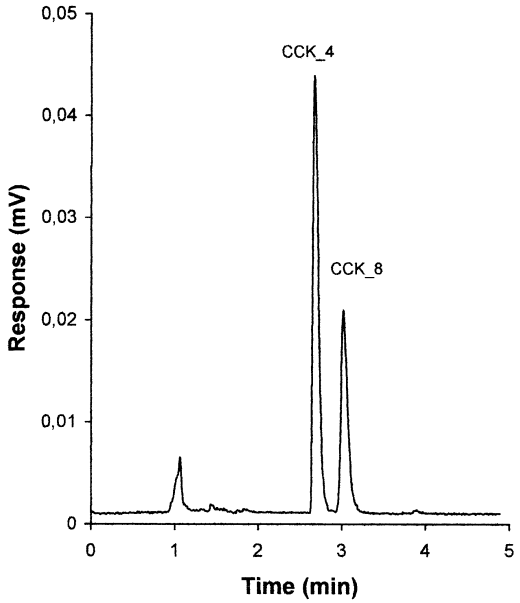

Figure 3. Separation of CCK-4 and CCK-8 fragments of cholecystokinin (Proportion 3 : 1) in the same analysis, with short analysis time (<5 min) achieving the required resolution and efficiency by RP-HPLC.

important for people working in this field, since the validity of the analytical procedure should be maintained whenever used.

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