Validation of a UV Spectrophotometric Method for the Determination of Melatonine in Solid Dosage Forms

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The aim of the work described in this paper was to provide a fast, easy, inexpensive, precise, and accurate method for the determination of melatonine in solid pharmaceutical dosage forms. The developed method is based on a UV first-derivative spectrophotometric determination, which exhibits excellent linearity in aqueous solutions ($r^2 = 0.996$) for analyte concentrations of 1.5–4.5 mg/dL within a pH range of 5–9. Neither excipients present in the formulation nor indole adulterants, such as tryptophan (up to 5%), interfere with the assay. A study of variation parameters showed that sonication temperature was the main factor for successful determination. At temperatures of <45°C, the sample dissolved completely, and accurate spectrophotometric measurements were obtained. A study was conducted of all the parameters established by the United States Pharmacopeia, 23rd Rev., to validate an analytical method for a solid pharmaceutical form, i.e., linearity, range, accuracy, precision, and specificity. All the parameters were in accordance with the acceptance criteria of the Comité de Guías Oficiales de Validación de la Dirección General de Control de Insumos para la Salud de Méjico. In addition, robustness and content uniformity tests were performed to substantiate the usefulness of the method.

Melatonine, a hormone secreted by the pineal gland in superior vertebrates, is truly considered a drug because of its physiological activity. It is generally used as a sleep inductor, an immune response-stimulating drug, and an antiaging agent (1).

Although there are various analytical methods to measure melatonine concentration, they are mainly biological assays that need low metabolite concentrations. The techniques used in these methods include gas chromatography with flame ionization detection and tandem mass spectrometry; liquid chromatography with various types of detection, such as fluorescence and UV–VIS spectrophotometric; and an enzymatic technique such as radioimmunoassay assay, with detection limits of <1 μg/mL (2–4). Although such methods are sensitive, they are time consuming and expensive, and therefore inadequate for a batch assay process in the pharmaceutical industry.

The major advantages of derivative spectroscopy, including high efficiency, flexibility, and speed of method development (5), prompted us to explore its application to the determination of melatonine in solid pharmaceutical forms.

Experimental

Apparatus

(**a**) *Spectrophotometer*.—UV–VIS Unicam (Cambridge, UK) UV-2-200, with 1 cm UV quartz cells.

(**b**) *Computer*.—486-DTK with Vision software (Cambridge, UK) version 2.11.

(**c**) *Water deionizer*.—Barnstead Thermolyne (Dubuque, IA)

(**d**) *Ultrasonic bath*.—Transonic Digital Elma (Singen, Germany)

(**e**) *Centrifuge*.—Heraeus Labofuge 400 (Hanau, Germany)

(**f**) *Buret*.—DIGITAL II (Wertheim, Germany) 50 mL/0.1 mL capacity.

(**g**) *Analytical balance*.—Precisa 40SM-200A (Zurich, Switzerland).

(**h**) *Micropipet*.—Variable volume, Transferpette (Werthein, Germany) 200–1000 μL.

(**i**) *pH meter*.—WTW Model pH 537 (Weilheim, Germany).

Reagents

(**a**) *Water*.—Deionized and purified (Milli-Q Plus; Molsheim, France).

(**b**) *Melatonine standard*.—99.5% purity (Maver Laboratories; Santiago, Chile).

(**c**) *Magnesium stearate*.—United States Pharmacopeia (USP; United States Pharmacopeial Convention, Inc., Rockville, MD).

(**d**) *Lactose*.—USP.

(**e**) *Sodium hydroxide solution*.—1M.

(**f**) *Britton-Robinson buffer*.—Prepared from equal volumes of 0.1M phosphoric acid, 0.1M acetic acid, and 0.1M boric acid.

Preparation of Standard and Sample Solutions

(**a**) *Melatonine standard methanol solution (1 mg/mL)*.—A 100 mg portion of melatonine standard was accurately weighed

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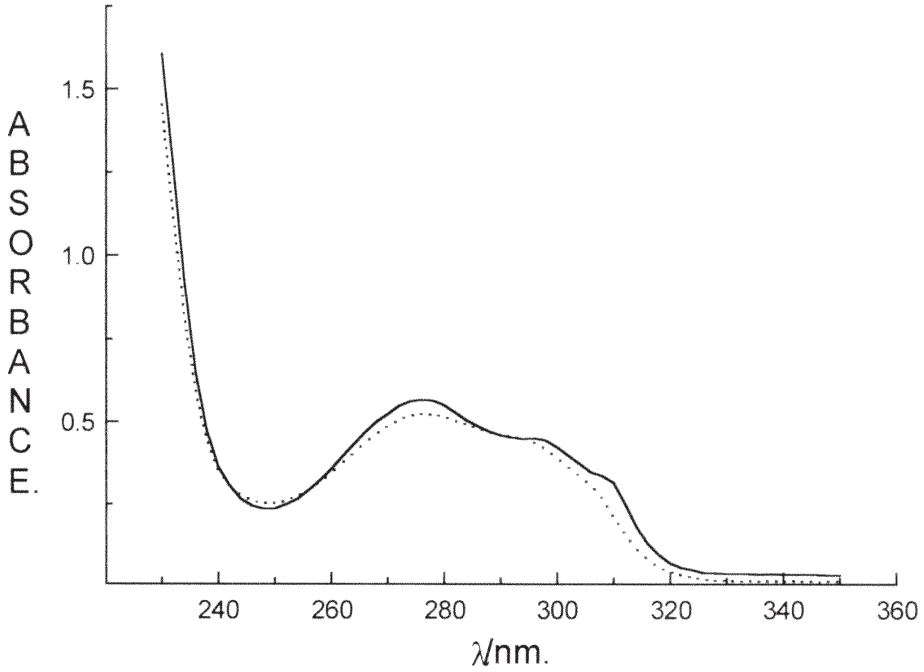

Figure 1. Ultraviolet spectra of 1 $\times 10^{-4}$ M solutions of melatonine in water (solid line) and methanol (dotted line).

and transferred to a 100 mL volumetric flask with methanol. The flask was shaken, and methanol was added to volume.

(**b**) *Melatonine standard aqueous solution (1 mg/mL).*—A 100 mg portion of melatonine standard was accurately weighed and transferred to a 100 mL volumetric flask, and purified water was added to bring the volume to 2/3 of the capacity of the flask. This standard solution was sonicated at 25°C for 15 min. Then purified water was added to final volume.

(**c**) *Melatonine standard aqueous solution (1 mg/mL) plus excipients.*—By means of the digital buret, precisely measured aliquots of the melatonine standard aqueous solution (1 mg/mL) were transferred to several volumetric flasks. An excipient mixture of 0.16 g lactose plus 8×10^{-4} g magnesium stearate was added to each flask. These samples were sonicated at 25°C for 15 min, and the contents of each flask were diluted to volume with purified water.

Spectrophotometric Measurements

The absorbance of solutions containing melatonine at 0.04 mg/mL in methanol and in water was spectrophotometrically measured in the UV range from 230 to 330 nm (Figure 1) with a scan speed of 240 nm/min, a data interval of 1 nm, and a bandwidth of 2 nm. The first-derivative spectra were obtained by instrumental electronic differentiation (Vision software) in the range of 264–286 nm. The values obtained from spectrophotometer readings were expressed in arbitrary units as a function of the distance from the positive peak to the negative peak. The upper and lower limits of the melatonine concentration range under study were selected from 50 to 150% of the nominal concentrations of actual samples and were expressed as mg/dL.

Determination of Parameters of Analytical Performance

(**a**) *Determination of linearity and range.*—Aliquots of melatonine standard aqueous solution were taken in triplicate.

Table 1. Determination of linearity and range[a]

Sample	Melatonine, mg/dL	Spectrophotometric reading, U^1D ($D_{264–286\ nm}$)
1	1.50	30.53
2	1.75	35.48
3	2.00	41.37
4	2.25	45.98
5	2.50	51.12
6	2.75	57.35
7	3.00	60.99
8	3.25	65.86
9	3.50	71.25
10	3.75	77.43
11	4.00	82.84
12	4.25	86.11
13	4.50	93.34

[a] First-derivative spectrophotometric values for a set of aqueous solutions of melatonine in the 1.5–4.5 mg/dL concentration range, exhibiting the linear regression equation Y = A + Bx, with the following values: A = 0.63 arbitrary $^1D_{264–286}$ ($U^1D_{264–286}$); B = 20.37 U^1D/mg/dL; r = 0.998; and r^2 = 0.996.

Table 2. Determination of precision[a]

Sample	Spectrophotometric reading, U^1D ($D_{264–286\ nm}$)	Melatonine, mg/dL
1	82.11	3.97
2	82.38	3.98
3	82.19	3.97
4	82.84	4.00
5	82.36	3.98
6	82.50	3.99
7	82.03	3.96

[a] Relative standard deviation = 0.34%.

Concentrations corresponding to 1.50, 2.28, 2.70, 3.30, 3.90, and 4.50 mg were transferred to separate 100 mL volumetric flasks, and the first-derivative values ($^1D_{264–286\ nm}$) for each sample were determined and recorded (Table 1).

(**b**) *Determination of precision*.—Several 2.0 mL aliquots of melatonine standard solutions were transferred with a volumetric pipet to 50 mL volumetric flasks, and the contents of each flask were diluted to volume with the appropriate solvent. These solutions were transferred to the sample cell of the spectrophotometer, and the first-derivative values were measured and recorded. Each test was repeated 7 times, with exactly the same protocol (Table 2).

(**c**) *Determination of accuracy*.—Aliquots were taken from melatonine standard solutions, starting at 1.50 mg with increments of 0.25 up to 4.5 mg. These aliquots were transferred in triplicate to 100 mL volumetric flasks and processed as previously described. The first-derivative values for each sample were measured spectrophotometrically ($^1D_{264–286\ nm}$) and recorded. A 10 mL aliquot from each of these solutions was transferred to a 50 mL volumetric flask containing 2 mg melatonine. The contents of each flask were carefully diluted to volume, and the first-derivative values ($^1D_{264–286\ nm}$) were measured and recorded (Table 3).

Table 3. Determination of accuracy[a]

Melatonin added, mg	Melatonin found, mg	Melatonin recovery, %[b]
1.50	1.50	100.0
1.75	1.74	99.4
2.00	2.00	100.0
2.25	2.25	100.0
2.50	2.50	100.0
2.75	2.78	101.1
3.00	2.99	99.7
3.25	3.23	99.4
3.50	3.49	99.7
3.75	3.79	101.1
4.00	4.06	101.5
4.25	4.22	99.3
4.50	4.57	101.6

[a] $n = 3$.

[b] Average recovery = 101.1%; relative standard deviation = 0.80%.

(**d**) *Determination of specificity*.—Mixtures containing decreasing percentages of melatonine (100 to 90%) and increasing percentages of tryptophan (0 to 10%) were prepared, and the melatonine content of each solution was determined (Table 4).

(**e**) *Determination of robustness*.—Several 2 mL aliquots of melatonine standard aqueous solution were analyzed individually with one of the following variations in the method parameters: pH (pH 5, pH 9), solvent (purified water, tap water), time of sample dissolution (5 min, 15 min), and temperature of sample dissolution (25°C, 45°C); the spectrophotometric readings ($^1D_{264-286\ nm}$) after each modification were recorded.

(**f**) *Assay of pharmaceutical samples*.—Three samples of finely ground powder from ≥20 tablets or ≥20 capsules, equivalent to ca 25 mg active ingredient, were accurately weighed. Each sample was quantitatively transferred with four 5 mL volumes of purified water to a suitable glass-stopper 25 mL volumetric flask. After 15 min of sonication at a temperature of ≤25°C, purified water was added to volume. A 1 mL aliquot of this solution was transferred to a 100 mL volumetric flask, and the solution was diluted to volume with purified water. Then a first-derivative spectrophotometric measurement ($^1D_{264-286\ nm}$) was obtained for each solution.

(**g**) *Content uniformity*.—Each of 10 pharmaceutical dosage forms (i.e., tablets or the contents of capsules) was transferred to a 200 mL flask, and deionized water was added. The sample was dissolved at 25°C with the aid of ultrasound for 15 min. After sonication, the solution was allowed to stand at room temperature for 15–30 min and then carefully diluted to volume with deionized water.

Then, a melatonine standard solution was prepared by weighing ca 80 mg melatonine and repeating the procedure, but with a 1 L volumetric flask.

From the first solution, six 25 mL aliquots were transferred to an equal number of 50 mL volumetric flasks. With a digital buret, aliquots of the melatonine standard solution were added to the flasks to obtain standard volumes in a 3–18 mL range with a difference of 3 mL from flask to flask. After standing for 15 min, the solutions were diluted to volume with deionized water.

The equation for the 6 points was determined; the abscissa was the standard volume, and the ordinate was $^1D_{264-286\ nm}$. The procedure was continued only if the linear correlation coefficient (r) was ≥0.99.

The intercept (a) and the slope (b) were applied to the following equation:

$$\frac{\text{Melatonine content}}{\text{dosage form}} = \frac{8 \times \text{Cst}}{\text{b}}$$

Table 4. Determination of specificity

Melatonine added, mg/dL	Tryptophan added, mg/dL	Melatonine found, mg/dL	Melatonine recovery, %
2	0	2	100
1.98	0.02	1.98	100
1.96	0.04	1.96	100
1.94	0.06	1.94	100
1.92	0.08	1.92	100
1.90	0.10	1.90	100
1.88	0.12	1.94	103

A similar procedure was followed with the remaining 9 pharmaceutical dosage forms, but $^1D_{264–286}$ nm was obtained by using 2 mL solution and additions of 0.2, 0.4, and 0.6 mL melatonine standard solution:

$$\frac{\text{Melatonine content}}{\text{dosage form}} = \frac{{}^1D_{264\text{-}286nm} \times Vst \times Cst \times 200}{{}^1D_{264\text{-}286nm} \times (2 + Vst) - 2 \times {}^1D_{264\text{-}286nm}}$$

where Cst is concentration of standard, and Vst is volume of standard.

A double-blind study of commercial pharmaceutical dosage forms from 6 laboratories was conducted by using the method described above.

Results and Discussion

Figures 1 and 2 show that the first-derivative spectra of solutions of pure melatonine in methanol and water are adequate for melatonine determination.

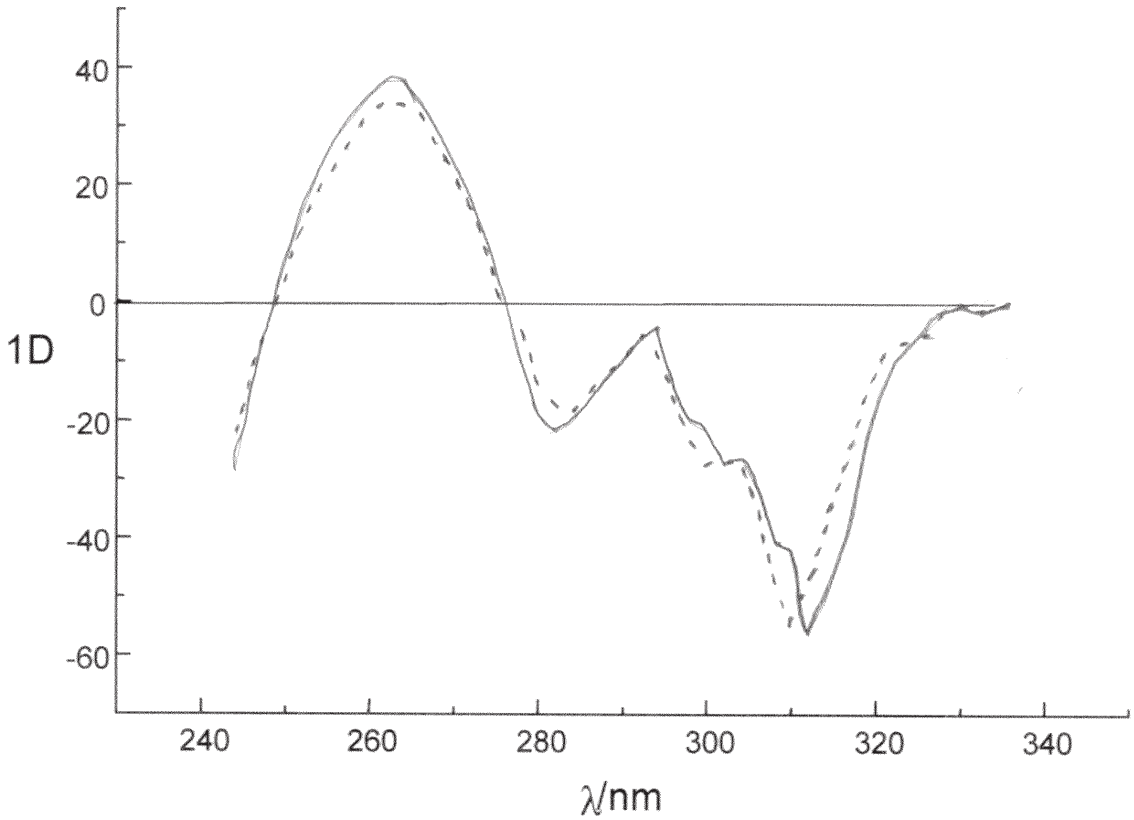

Figure 2. Ultraviolet first-derivative spectra of 1 $\times 10^{-4}$M solutions of melatonine in water (solid line) and methanol (dotted line), showing positive and negative ordinates at 264 and 286 nm, respectively.

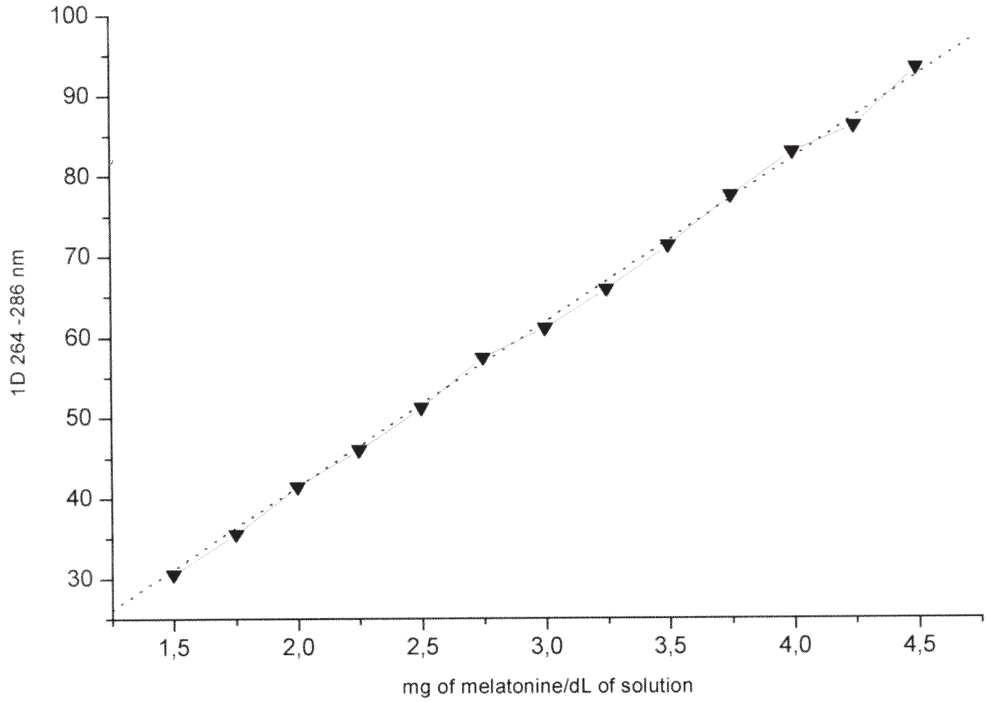

Figure 3. Linearity of $^1D_{264–286\ nm}$ versus concentration of melatonin in aqueous solution; dotted line = regression curve; solid line = experimental curve.

According to the data reported by Shida et al. (6), melatonine is soluble in a purely aqueous medium, and its concentration in this medium can be as high as 5×10^{-3}M. Such concentrations are adequate for melatonine determinations in aqueous solutions by spectrophotometric methods.

The parameters for validating an analytical method for a solid pharmaceutical form were obtained, and the acceptance criteria were met (7–11) as follows: (*1*) The linearity of the spectrophotometric response over the 50–150% concentration range of the actual melatonine concentration (i.e., 1.5–4.5 mg/dL) was examined. Linear plots of spectrophotometric first-derivative readings ($U^1D_{264–286\ nm}$) versus the absolute concentrations of melatonine yielded a linear squared regression coefficient (r^2) of 0.99 that met the requirement established for the standards of quality considered in this study, $r^2 \geq 0.98$. A nonzero intercept [$Y_{(U1D)} = 20.37_{U1D} \times C$ (mg/dL) + 0.63_{U1D}] occurred in the plot of the U^1D spectrophotometric readings versus concentrations of melatonine. This was nonsignificant with respect to the accuracy of the measurements at a concentration of 3 mg/dL (Table 1; Figure 3). (*2*) Precision determined as the relative standard deviation (RSD) for 7 aliquots of 4 mg/dL melatonine solution plus excipients was 0.34%. The RSD of 0.34% easily fulfills the accepted criterion of ≤3.00% (Table 2). (*3*) Accuracy is expressed as the percentage of melatonine recovered by the assay for the known amount of analyte added (procedure already described). The results obtained for samples analyzed in triplicate and containing added amounts of melatonine ranging from 1.5 to 4.5 mg (Table 3) show that the average recovery of the drug for 13 samples processed is 100.1%, which is within the acceptance range of 97–103%. (*4*) Specificity was expressed as percent melatonine recovered from a mixture containing tryptophan added as an impurity in different proportions. No significant change was found in melatonine recovery when the concentration of tryptophan was <5% (Table 4). The values obtained under those conditions exceeded the criterion for an acceptable loss in recovery as reported in the literature (1%).

To complement the analytical profile, determination of robustness by varying experimental conditions, i.e., pH, solvent, time of sample dissolution, and temperature of sample dissolution, established that the factor that most affected the precision of the method was the temperature generated by the ultrasound treatment during the dissolution process. We concluded that temperature should remain below 45°C; otherwise the method would produce results with variations of >10%. The other factors studied such as pH, solvent, and processing time were of no analytical significance.

The traditional excipients of a capsule or tablet, like lactose and magnesium stearate, are transparent in the spectroscopic ultraviolet region, and the existence of some turbidity does not interfere with the first-derivative spectrophotometric determination.

Because extraction and filtration were unnecessary, there was no loss of drug by sample treatment. In addition, when this method was applied to commercial samples in a double-blind study of content uniformity, only 2 of them met the USP requirements.

In conclusion, this method will determine melatonine in aqueous solutions within the range of 1.0–4.5 mg/dL without extraction and filtration; the method can also be used for the analysis of authentic samples containing ≤5% indole impurities.

Acknowledgment

We express our gratitude to C. Telha (University of Chile) for his valuable assistance in the English revision of this paper.

References

(1) Brzezinski, A. (1997) *N. Engl. J. Med*. **336**, 186–195

(2) Svendsen, H., & Greibrokk, T. (1981) *J. Chromatogr*. **212**, 153–166

(3) Brinklow, B., & Rodway, R. (1982) *J. Physiol*. (London) **330**, 22

(4) Leone, R., Silan, R., Hooper, R., Finnie, M., Smith, I., Francis, P., Mullen, P., Linsell, C., Savage, M., & Preece, M. (1979) *J. Endocrinol*. **83**, 47–48

(5) Schmitt, A. (1977) in *Derivative Spectroscopy, An Introduction with Practical Examples, Applications Data Bulletin*, Perkin-Elmer, Norwalk, CT

(6) Shida, C.S., Castrucci, A.M., & Lamy-Freund, M.T. (1994) *J. Pineal Res*. **16**, 198–201

(7) Castro, M., Gascón, S., Pujol, M., Sans Roca, J., & Vicente, L. (1989) in *Validación de Métodos Analíticos*, Asociación Española de Farmacéuticos de la Industria, Sección Catalana, Comisión de Normas de Buena Fabricación y Control de Calidad, Barcelona, Spain, pp 4–66

(8) Pasteelrick, L. (1993) in *Analytical Methods Validation, In Pharmaceutical Process Validation*, 2nd Ed., I. Berry, & R. Nash (Eds), Marcel Dekker, New York, NY, pp 411–428

(9) *Guidelines on General Principles of Process Validation* (May 1987) Division of Manufacturing and Product Quality (HFN-320), Center for Drugs and Biologics, U.S. Food and Drug Administration, Rockville, MD

(10) *The United States Pharmacopeia* (1995) 23rd Rev., United States Pharmacopeial Convention, Inc., Rockville, MD

(11) Métodos Analíticos: *Validación* (1992) Comité de Elaboración de Guía Oficiales de Validación de la Dirección General de Control de Insumos para la Salud, SSA, México City, Mexico