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Validation of an analysis method for 4-amino-3-hydroxybutyric acid by reversed-phase liquid chromatography

M. Candela, A. Ruiz*, F.J. Feo

FAES, S.A. Department of Quality Control, Máximo Aguirre 14, E-48940 Lamiaco-Lejona, Spain

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Abstract

A rapid and simple reversed-phase liquid chromatographic method that did not require the derivatization of 4-amino-3-hydroxybutyric acid (GABOB) was developed and validated. The method proved to be suitable for the determination of GABOB concentrations in finished pharmaceutical product (tablets). The method was developed using a RP-18 column, UV detection at 210 nm and 0.01 *M* sodium heptasulphonate solution, at pH 2.4, as the mobile phase. Different validation parameters were included and satisfactorily evaluated. The specificity of the method was demonstrated. Linearity was established in the range 0.40–0.60 mg/ml ($r=0.997$). The method showed excellent accuracy (100.1%). Precision (repeatability) gave a relative standard deviation value of 0.68%, while the intermediate precision was 1.70%. A robustness test showing the influence of different pH values and counter-ion concentrations was also performed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: 4-Amino-3-hydroxybutyric acid

1. Introduction

One essential part of Good Manufacturing Practices in the pharmaceutical industry is analytical method validation. It is the process for establishing that performance characteristics of the method, expressed in terms of analytical parameters, are suitable for the intended application. Chromatographic methods play an important role in the pharmaceutical field and, hence, they need to be validated when they are developed and intended to be for routine use.

4-Amino-3-hydroxybutyric, or γ-amino-β-hydroxybutyric acid (GABOB), is an amino acid used for treating hemiplegia, memory and speech disturbances, essential neurogenous hypertension, epilepsy and arrested mental development. Reversed-phase chromatographic analysis of amino acids usually includes a prior derivatization process with different derivatization agents (e.g., phenylisothiocyanate) [1–5]. These determinations are tedious and time-consuming. No high-performance liquid chromatographic (HPLC) method without derivatization for GABOB determination has been reported.

*Corresponding author. Tel.: +34-944-639-000; fax: +34-944-800-547.

E-mail address: aruiz@faes.es (A. Ruiz).

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The aim of this work was to develop and validate a new, simple and rapid method for the quantitative analysis of GABOB in a finished pharmaceutical product by reversed-phase chromatography.

2. Experimental

2.1. Instrumental and operating conditions

The HPLC analysis was carried out on a Merck–Hitachi La Chrom® liquid chromatograph with an autosampler Model L-7200 and an L-7100 pump connected to a photodiode-array detector, L-7450A (Merck–Hitachi, Darmstadt, Germany). The column used was a reversed-phase C_{18} Purospher (25×4 mm I.D.; 5 μm; Merck, Darmstadt, Germany), thermostated at 40°C. The mobile-phase composition was 0.01 *M* sodium heptanesulphonate, at pH 2.4, with H_3PO_4 and the flow-rate was set at 1.0 ml/min. The injection volume was 50 μl and the detection wavelength was set at 210 nm. The instrument and chromatographic data were fully managed by the D-7000 HPLC System Manager Software (Merck–Hitachi).

A Perkin-Elmer high-performance liquid chromatograph equipped with an autosampler (model ISS 200), a Series 200 LC quaternary pump and an LC-235 diode array detectorn operating at 210 nmn was used for the study of the intermediate precision. All of the equipment's functions and data obtained were managed by the Turbochrom Workstation program (PE Nelson). Column, mobile phase, flow-rate, injection volume and detection wavelength were the same as the chromatographic conditions used in the Merck–Hitachi HPLC system.

2.2. Reagents and materials

DL-γ-Amino-β-hydroxybutyric acid (Sigma, St. Louis, MO, USA) was used to prepare the standard solutions. Phosphoric acid (85%) was purchased from Merck and sodium heptanesulphonate (HPLC grade) was from Scharlau (Barcelona, Spain). 5-Ethyl-5-phenylbarbituric acid (phenobarbital) was obtained from ICN Hungary (Tiszavasvári, Hungary), HCl pyridoxine was from F. Hoffmann La Roche (Basel, Switzerland), phenytoin (diphenylhydantoin) was from Recordati (Milan, Italy), microcrystalline cellulose M-101, was from Mingtai Chemicals (Taipei, Taiwan) and magnesium stearate was from Mallinckrodt (St. Louis, MO, USA). HPLC ultrapure water was generated by a Sation 9000 System Aqualab (Barcelona, Spain). The mobile phase was filtered through a 0.45-μm membrane (Millipore, Bedford, MA, USA). All samples were filtered through a hydrophilic Durapore-PVDF membrane of 0.45 μm pore size (4 mm Millex-HV, Millipore, Yonezawa, Japan) before injection.

2.3. Sample preparation

2.3.1. Preparation of stock standard solution

A 250-mg quantity of DL-γ-amino-β-hydroxybutyric acid was accurately weighed and dissolved in 25 ml of water, followed by the addition of mobile phase to 50 ml.

2.3.2. Preparation of standard solutions

Standard solutions were prepared by the dilution of the stock standard solution with mobile phase to five different concentrations over the range of interest, in this case, 0.40, 0.45, 0.50, 0.55 and 0.60 mg/ml, corresponding to 80, 90, 100, 110 and 120% of labelled concentration, respectively. A concentration of 0.50 mg/ml is considered the labelled concentration, being the GABOB concentration obtained after the extraction procedure from the tablets (one tablet in 200 ml of water was mechanically shaken for 30 min and sonicated in a water bath for 5 min, to make sure of its total dissolution).

2.3.3. Preparation of standard solution with placebo

Placebo formulation was added to the different standard solutions in an equivalent amount to the one obtained by dissolving one tablet in 200 ml of water. Standard solutions with placebo samples must be filtered through 0.45 μm filters before injection.

Placebo formulations contained 5-ethyl-5-phenylbarbituric acid, pyridoxine, phenytoin, microcrystalline cellulose pH 101 and magnesium stearate.

3. Validation of the method

The procedures and parameters used for the validation of the chromatographic method developed in this study are the ones described in the U.S. Pharmacopoeia (USP) 24, chapter (1225) [6] and ICH-Guidelines [7]. Results were processed using the Validation Manager Software (Version 1.3., Merck, France).

3.1. Specificity

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. Other reputable international authorities (IUPAC, AOAC) have preferred the term selectivity, reserving specificity for those procedures that are completely selective [6].

According to Persson et al. [8], a common and serious mistake is referring to a method as specific when it is only selective. This case is especially true when dealing with chromatographic methods that are not absolute, but only relative, methods of analysis [8]. According to the USP terminology and ICH guidelines, and to avoid any misunderstanding, we decided to use the term specificity when referring to this validation parameter.

Demonstration of specificity requires the ability of the method to show that the procedure is not interfered with by the presence of impurities or excipients expected to be in the sample matrix. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities/excipients and demonstrating that the assay result is unaffected by the presence of these potential interferences [6].

The specificity of the analytical method in this study was determined by comparing the results from the analysis of samples containing degradation products or placebo ingredients with those obtained from the analysis of samples containing only 4-amino-3-hydroxybutyric acid. The analyzed samples were GABOB standard, placebo formulation spiked with GABOB, and a tablet that was stressed at 60°C in an oven for 18 h.

Fig. 1 shows the chromatograms of the different samples. The analyte of interest was well separated from other components present in the samples. The peak purity was, in all cases, more than 99.0%.

3.2. Linearity

The linearity of an analytical method is its ability to produce test results that are directly proportional to the concentration of analyte in samples within a given range. For the establishment of linearity, a minimum of five different concentrations should be used. It is also recommended that a specific range, normally from 80 to 120% of the test concentration, is used for the assay of a finished pharmaceutical product.

A linear curve fit was obtained from five different concentrations of standard solutions in the range 0.40 to 0.60 mg/ml using three replicate injections. The regression line was calculated as $y = a + bx$, where x was the GABOB concentration (mg/ml) and y was the response (peak area expressed as AU). The calibration curve was obtained using the linear least squares regression procedure. Fig. 2 shows the regression line and Fig. 3 a standard chromatogram.

The RSD (relative standard deviation) values for the response factors were in the range 0–5% (1.7%), and they were considered adequate for verifying the linearity of the regression [9].

The coefficient of correlation (r) value was close to unity (0.997), hence, there was a linear relationship between the amount of GABOB and the detector response.

3.3. Accuracy

Accuracy is the closeness of test results obtained by the method to the true value. Accuracy is often calculated as percent recovery by the assay of known, added amounts of analyte to the sample [6]. The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering a specific range (i.e., three concentrations and three replicates for each concentration) [7].

Accuracy was determined by spiking known amounts of analyte to a placebo sample across the specified range of the analytical procedure to obtain

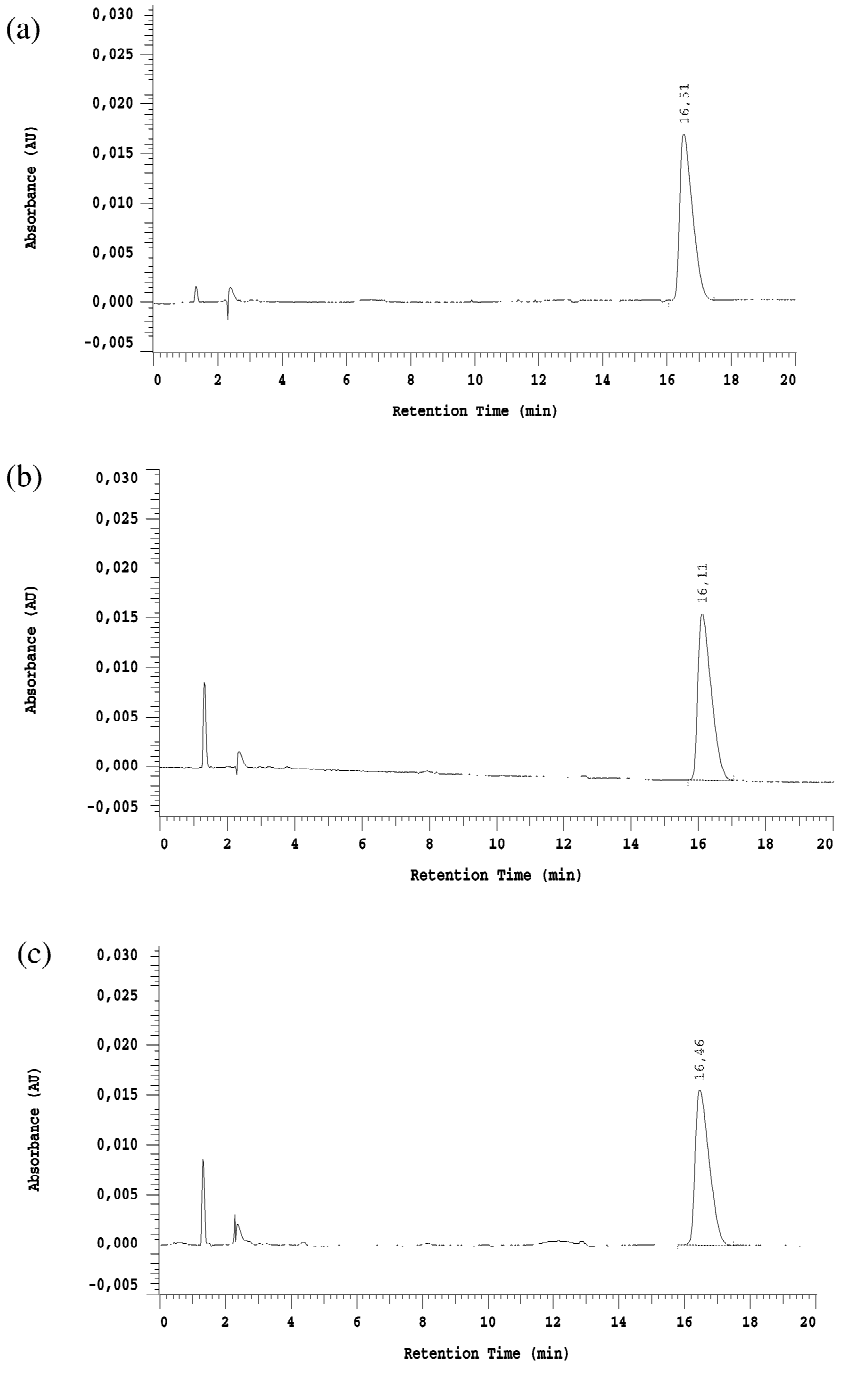

Fig. 1. Specificity chromatograms. (a) 4-Amino-3-hydroxybutyric acid standard, (b) placebo formulation and 4-amino-3-hydroxybutyric acid, (c) a stressed tablet at 60°C for 18 h.

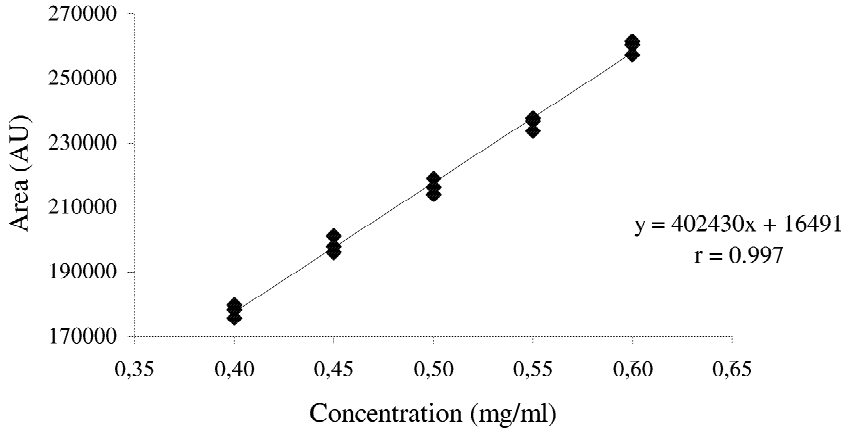

Fig. 2. Calibration curve.

0.40, 0.50 and 0.60 mg/ml concentrations (80, 100 and 120% of test concentration). Table 1 summarises the accuracy results, expressed as percent recovery and relative standard deviation (RSD). The method showed excellent recovery, with values that were close to 100% (100.1%).

3.4. Precision

The USP defines precision as 'the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of an homogeneous sample' [6]. Precision may be measured as repeatability, reproducibility and intermediate precision. Reproducibility refers to the use of analytical procedure in different laboratories, i.e., in a collaborative study. In this case, we have studied repeatability and intermediate precision.

3.4.1. Repeatability

The repeatability of an analytical method refers to the use of the procedure within a laboratory over a short period of time, carried out by the same analyst with the same equipment. The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range (i.e., three concentrations and three replicates for each concentration) or a minimum of six determinations of 100% of the test concentration [7].

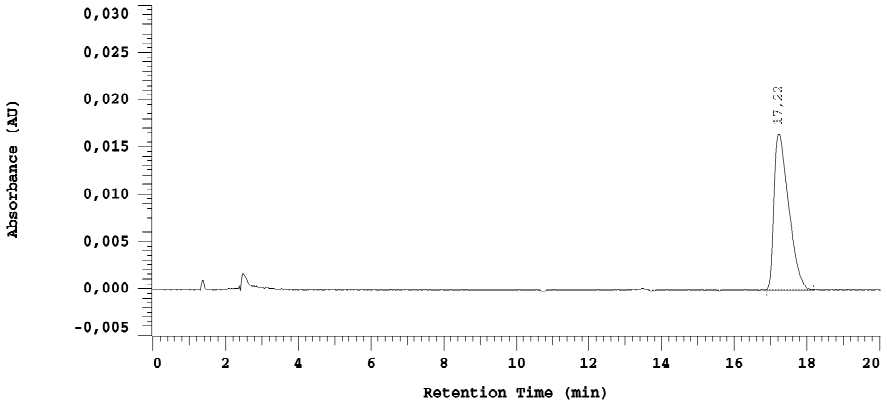

Fig. 3. Standard chromatogram (100% of labeled concentration).

Table 1
Results of accuracy determination[a]

Theoretical concentration (mg/ml)	Experimental concentration (mg/ml)	Recovery (%)
0.40	0.4029	100.72
0.40	0.4032	100.80
0.40	0.4019	100.47
0.50	0.4977	99.54
0.50	0.4969	99.38
0.50	0.5041	100.82
0.60	0.5958	99.30
0.60	0.5970	99.50
0.60	0.6011	100.18
	$\bar{x}$	100.1
	%R.S.D.	0.4

[a] Each number represents individual HPLC measurements.

We considered nine determinations covering the specified range for our procedure. Standard solutions of 0.40, 0.50 and 0.60 mg/ml spiked into placebo samples were injected (three replicates each) and the method was calibrated with three standard solutions (0.40, 0.50 and 0.60 mg/ml). The RSD value (0.68%) indicates that the proposed HPLC method shows acceptable repeatability (Table 2).

3.4.2. Intermediate precision

The intermediate precision of an analytical method is the degree of agreement of test results obtained by the analysis of the same sample under various conditions (typical variations include different laboratories, analysts, equipment, etc.) [10]. The extent this parameter should be studied depends on the circumstances under which the procedure is intended to be used [7]. The most interesting study in our Quality Control Laboratory is to check the viability of the proposed method using different equipment. The extraction procedure, column, mobile phase and the rest of the conditions were the same. The samples injected into the second HPLC system (Perkin-Elmer) were also the same as the ones used for the repeatability study. The results obtained by the two HPLCs were comparable. Table 3 presents the individual values obtained for the intermediate precision study. The results obtained from both HPLC systems were similar, although a slight variation was observed at the 120% level. The RSD value (1.27%) indicates that the proposed HPLC method shows acceptable intermediate precision.

Table 2
Results of repeatability determinations[a]

Theoretical concentration (mg/ml)	Experimental concentration (mg/ml)
0.40	0.4029
0.40	0.4032
0.40	0.4019
0.50	0.4977
0.50	0.4969
0.50	0.5041
0.60	0.5958
0.60	0.5970
0.60	0.6011

[a] RSD: 0.68%. Each number represents individual HPLC measurements.

3.5. Range

The range of an analytical method is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with acceptable precision, accuracy and linearity using the procedure, as written [6]. The method developed in our laboratory showed its suitability for GABOB determinations in the concentration range from 0.40 to 0.60 mg/ml.

3.6. Stability of solutions

The stability of solutions was demonstrated by analysing the 100% test concentration (0.50 mg/ml) and the same spiked into placebo just prepared and

Table 3
Results of intermediate precision determinations[a]

Theoretical concentration (mg/ml)	Experimental concentration (mg/ml) HPLC 1[b]	Experimental concentration (mg/ml) HPLC 2[c]
0.40	0.4029	0.4013
0.40	0.4032	0.4008
0.40	0.4019	0.3997
0.50	0.4977	0.4945
0.50	0.4969	0.4907
0.50	0.5041	0.4947
0.60	0.5958	0.5837
0.60	0.5970	0.5849
0.60	0.6011	0.5847

[a] RSD: 1.7%. Each number represents individual HPLC measurements.
[b] HPLC1: samples analysed using a Merck–Hitachi La Chrom® high-performance liquid chromatograph.
[c] HPLC2: samples analysed using a Perkin-Elmer high-performance liquid chromatograph.

after 6 days of refrigerated storage. The solutions did not undergo any decomposition. The results of the system suitability test and area values did not show significant differences.

3.7. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters, and provides an indication of its reliability during normal usage. Different types of designs are used in robustness testing, and ICH guidelines recommend its evaluation during the development phase of a method, and not at the end, as occurred in this study [11].

We analysed the influence of different pH values and mobile phase counter-ion concentrations. pH variations of the mobile phase produced important changes in the retention time and peak shape, while the counter-ion concentration did not show any significant change in the system's suitability parameters (retention time, capacity factor k', asymmetry and number of theoretical plates). The results are shown in Tables 4 and 5. The values presented are the average of two different measurements.

4. Conclusion

We developed a novel and straightforward analytical method for the determination of 4-amino-3-hydroxybutyric acid concentrations in pharmaceutical tablets. The main advantage of this HPLC procedure over the previous ones described for the same compound is that it does not require derivatization.

We studied the validation parameters according to USP and ICH recommendations and demonstrated that the proposed new method is specific, linear, accurate and precise, within the established range.

Therefore, the HPLC procedure developed was

Table 4
Results of robustness (effect of counter-ion concentration in the mobile phase) study[a]

Samples	Counter-ion concentration	Retention time (min)	k'(capacity factor)	Asymmetry	N (number of theoretical plates)	Area
GABOB	0.009 *M*	16.71	10.94	1.54	7346	224 240
standard	0.01 *M*	17.26	12.28	1.79	8049	222 660
	0.011 *M*	16.29	10.64	1.55	7490	218 448
Placebo	0.009 *M*	17.27	11.70	1.57	7444	22 488
+	0.01 *M*	17.22	11.30	1.72	8001	224 745
GABOB	0.011 *M*	16.41	10.73	1.58	7615	219 450

[a] Each value represents the average of two injections.

Table 5
Results of robustness (effect of mobile phase pH) study[a]

Samples	pH	Retention time (min)	k' (capacity factor)	Asymmetry	N (number of theoretical plates)	Area
GABOB	2.0	13.29	8.48	1.20	6780	212 138
standard	2.4	17.26	12.28	1.79	8049	222 660
	3.0	19.28	12.77	0.89	5762	171 100
Placebo	2.0	13.08	8.33	1.10	6389	215 665
+	2.4	17.22	11.30	1.72	8001	224 745
GABOB	3.0	19.29	12.78	0.85	5249	164 748

[a] Each value represents the average of two injections.

proved to be suitable for the determination of GABOB concentrations in pharmaceutical finished products.

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