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Determination of cyanocobalamin, betamethasone, and diclofenac sodium in pharmaceutical formulations, by high performance liquid chromatography

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Abstract

The aim of this work was to develop an analytical method for a simultaneous determination of cyanocobalamin (Vitamin B12), betamethasone, and diclofenac, present in pharmaceutical formulations, by high performance liquid chromatography, assuring rapidity, accuracy, precision, and selectivity. The working conditions were as follows: RP18 column of 125 mm × 4 mm ID and a particle size of 5 μm; mobile phase acetonitrile–water (40:60; v/v) (pH* 3.45) adjusted with acetic acid; flow gradient from 0.8 to 1.9 ml/min.; injection volume of 20 μl; temperature 34°C and detection at 240 nm. The method was adequately validated, and linearity, accuracy, as well as the system, method and interday precision, for each active principle, were determined.

Keywords: Cyanocobalamin; Betamethasone; Diclofenac sodium; High performance liquid chromatography; Validation

1. Introduction

The combination of cyanocobalamin (CC), betamethasone (BM) and diclofenac sodium (DC) is particularly effective for treatment of rheumatoid arthritis (Fig. 1). The action of DC, a powerful analgesic, antipyretic and anti-inflammatory non-steroid agent, and that of CC, used for treatment of trigeminal neuralgia and multiple sclerosis, are added to the anti-inflammatory action of BM, a glucocorticoid derived from cortisol [1–7].

When this combination of active principles is used in pharmaceutical formulations, a problem may occur to the pharmaceutical analyst in charge of quality control. If those active principles are in small concentrations, extreme precautions should be taken in the selection of the more advantageous analytical method.

To solve this problem through the application of a rapid method, but meeting the proper de-

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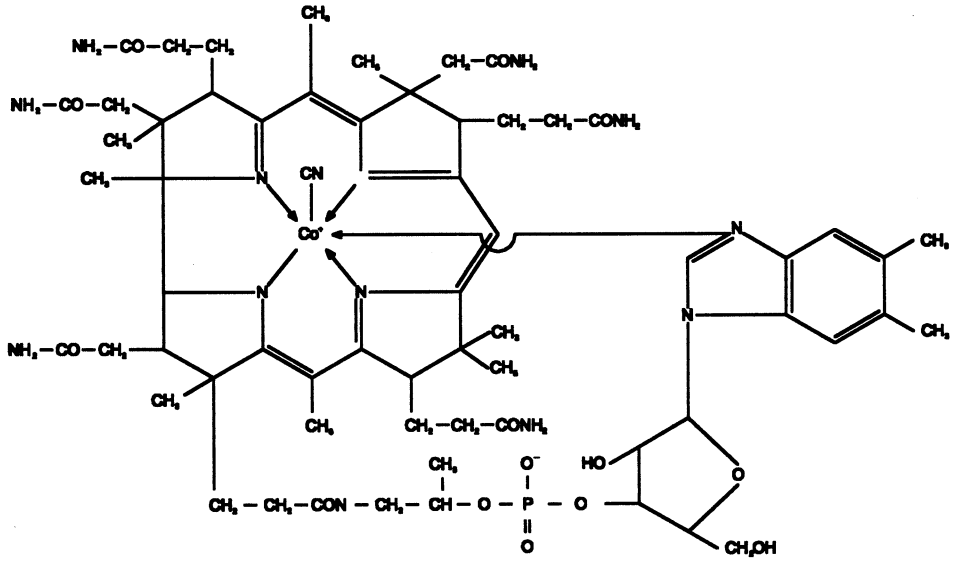
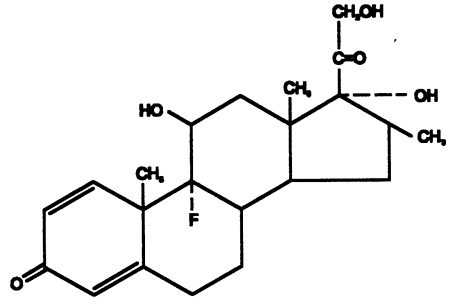
mands of quality control (accuracy, precision, sensitivity, and specially selectivity), when more than one active principle is involved, as in this case, a deep analytical development is required.

A lot of work about analytical methods developed for CC [8–12], BM [13–17] and DC [18–21] control, alone or in a combination with other active ingredients have been published. However, we have not found any method for the combination, which is the object of the present work.

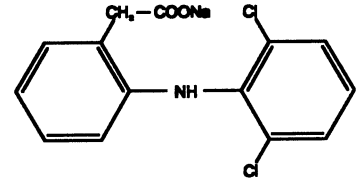
In the present work, a method using high performance liquid chromatography (HPLC) was developed, adequately validated to determine the content and dissolution kinetics of CC, BM and DC, present in tablets available in the pharmaceutical market, in order to carry out their chemical and biopharmaceutical control.



(a) Cyanocobalamin.



(b) Betamethasone.



(c) Diclofenac Sodium.

Fig. 1. Structures of (a) cyanocobalamin, (b) betamethasone and (c) diclofenac sodium.

2. Materials and methods

2.1. Equipment

The chromatographic system consisted of a Konik KNK 500G chromatographer, with a double-piston serial pump, equipped with a programmer for the microprocessor KNK 029-375 (Konik, Barcelona, Spain), a Rheodyne 7125 sample injector with a fixed loop of 20 μl capability (Rheodyne, Cotati, CA, USA), and a Helium bubbling degasificator KNK 029-254. Also a Lichrocart RP-18 reversed phase column of 125 mm × 4 mm ID and a particle size of 5 μm was used (Merck, Darmstadt, Germany), with a precolumn of 50 mm × 4.6 mm ID, packed with 40 μm Pelliguard LC-18 (Supelco, Bellefonte, PA, USA). A variable wavelength UV–visible detector, model 204 (Linear, Nevada, USA), was used, and the integrator was a Datajet model SP 4600 (Spectra Physics, San José, CA, USA).

For the preparation of samples, a Socorex micropipette of 100–1000 μl was used (Socorex, Switzerland); drugs and reagents were weighted on a Metler Toledo AG 204 balance (Metler, Greinfensee, Switzerland), and dissolution profiles were obtained using the dissolution equipment Sotax AT7 (Sotax AG, Basel, Switzerland).

2.2. Materials

Cyanocobalamin, Betamethasone and Diclofenac sodium, powdered drugs, USP 23 degree. Tablets from the pharmaceutical market, containing CC, BM and DC, identified as Laboratory A, B and C.

Acetonitrile, HPLC grade (Merck), sterile water for injection (Roux Ocefa, Buenos Aires, Argentina); glacial acetic acid, RSE (Erba, Milano, Italia).

Millipore membrane type HV with a diameter of 0.45 μm and 50 mm (Millipore, Bedford, MA, USA), and MSI with a diameter of 0.45 μm and 13 mm (MSI, Westborough, MA, USA), were used to filter the mobile phase and the samples, respectively.

2.3. Chromatographic conditions

The mobile phase was acetonitrile–water (40:60, v/v) (pH* 3.45) adjusted with acetic acid, in order to eliminate the tailing presented by DC.

The flow required a gradient from t_0 to $t_{6.5}$ of 0.8 ml/min, and from $t_{6.5}$ of 1.9 ml/min; in this way, the DC retention time, which is the component retained in the column, was improved.

Attenuation (AT) also required a gradient, since the concentrations of active principles in the pharmaceutical formulations containing them were very different; from t_0 to $t_{6.5}$, the AT was 32, from $t_{6.5}$ it was 256.

The integration needed a baseline correction, during the initial time, due to a negative peak of the mobile phase that can be integrated together with CC.

The injection volume was 20 μl, the temperature was 34°C, and the active principles were monitored at 240 nm.

2.4. Standards preparation

The standards of CC and DC were prepared in mobile phase, in all the dilutions. BM needed to be completely solubilized in acetonitrile, and then the pertaining dilutions were performed in the mobile phase.

2.5. Construction of the calibration curve

The concentration range was made respecting the relation in which the active principles are found in pharmaceutical formulations: 5 mg of CC, 0.3 mg of BM, and 50 mg of DC.

Five replications of the calibration curve were obtained, with four or five concentrations for each one. For CC, the following concentrations were used: 5, 10, 15, 20, 25 and 30 μg/ml; for BM, 0.5, 1.0, 1.5 and 2.0 μg/ml; and for DC, 90, 150, 240 and 300 μg/ml.

2.6. Sample preparation

The tablets analyzed were treated individually, disintegrating them in a small volume of mobile phase, then completing at 25 ml, and shaking

magnetically during 30 min; after filtering and diluting 3:25 with mobile phase. Solutions having concentrations of 24 μg/ml of CC, 1.44 μg/ml of BM and 240 μg/ml of DC were obtained . These concentrations fell within the calibration curve, and were injected three times into the chromatographer.

3. Results and discussion

Fig. 2 shows a typical chromatogram of the separation of these three active principles, under the specified conditions. The retention times were 1.50 min for CC, 4.46 min for BM, and 16.21 min for DC.

3.1. Linearity

A linear response was observed for CC in a concentration range of 5–30 μg/ml, verifying the value of area/100.000, with a coefficient of correlation $r = 0.9984$. The intercept (a) and the slope (b) with the respective confidence intervals of 95% were: $a = -0.164 \pm 0.1$, $b = 0.366 \pm 0.006$.

BM showed linearity for a range of 0.5–2.0 μg/ml (area/10.000), with $r = 0.9983$, $a = 0.0165 \pm 0.18$ and $b = 8.884 \pm 0.13$.

DC showed a linear response for a range of 90–300 μg/ml (area/1.000.000), $r = 0.9974$, $a = -0.041 \pm 0.1$, and $b = 0.022 \pm 0.0004$.

In order to confirm the regression analysis and the linearity, an analysis of the response factor was carried out, i.e. relation between the signal (in this case area) and concentration, which should be maintained constant along the range of concentrations of the calibration curve.

In the same sense, we employed Bartlett's test for determining the overall data homoscedasticity. Differences between observed and critical values evaluated by means of the χ^2 test were not significant ($P < 0.05$).

These analyses confirmed that the model used for this analytical method was linear [22].

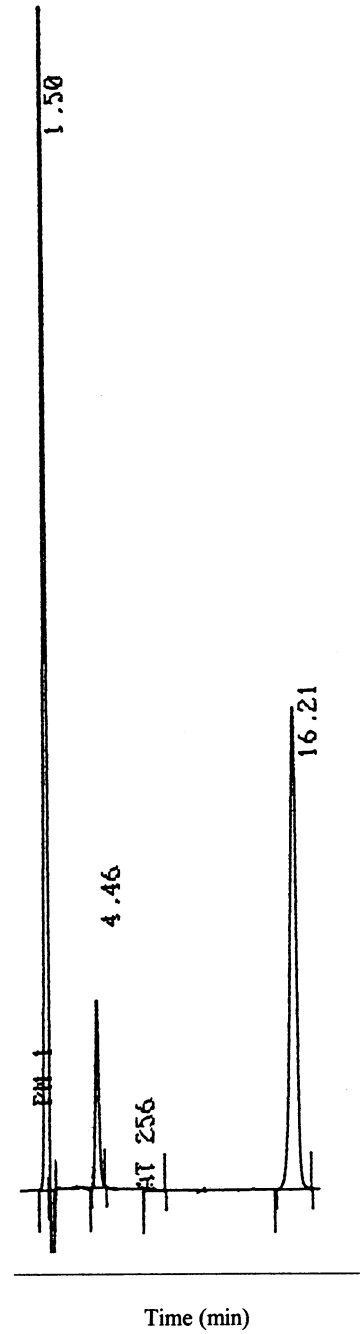


Fig. 2. Typical chromatogram of a sample of cyanocobalamin (1.5 min), betamethasone (4.46 min) and diclofenac (16.21 min).

3.2. Precision

Three levels of precision were obtained; (a) the system precision calculated as the coefficient of variation (CV) of eight injections of the same

Table 1
Precision for CC, BM and DC, expressed as CV (%)

Precision (CV)	Cyanocobalamin	Betamethasone	Diclofenac
System ($n = 8$)	0.76	0.72	0.92
Method ($n = 5$)	0.52	0.65	1.86
Interday ($n = 4$)	1.75	3.3	1.27

Table 2
Assay of recovery for each active principle

($n = 5$)	% Recovered ± CV
Cyanocobalamin	101.7 ± 0.33
Betamethasone	102.8 ± 1.02
Diclofenac	100.68 ± 0.64

solution of the standard on the same day; (b) the method precision calculated as the CV of five samples of the same standard; and (c) the interday precision calculated as the CV of the same standard injected on four different days. The results obtained are shown in Table 1.

3.3. Accuracy

In order to determine the accuracy, an assay of recovery was performed, using the excipients of one pharmaceutical formulation, to which the composition was known; a blank of excipients allows us to prove that no interference was produced, and therefore the method was specific with respect to them. The results obtained are shown in Table 2.

Table 3
Percentage of the labeled amount of each active principle ± CV

($n = 5$)	Cyanocobalamin	Betamethasone	Diclofenac
Laboratory A	104.8 ± 2.22	92.3 ± 3.20	98.6 ± 2.30
Laboratory B	110.0 ± 4.06	76.7 ± 3.07	100.6 ± 0.83
Laboratory C	103.9 ± 1.89	63.2 ± 1.83	94.7 ± 1.70

3.4. Tablet control

Three pharmaceutical formulations available on the market were controlled, which were identified as Laboratory A, B and C, containing this association with the same formula. The results are shown in Table 3.

3.5. Dissolution study

The dissolution study was carried out using 500 ml of water as the dissolution medium, the paddle method, an agitation speed of 50 rpm, a tempera-

Table 4
Mean percentages ($n = 6$) dissolved of each active principle

Time (min)	% Dissolution								
	Laboratory A			Laboratory B			Laboratory C		
	CC	BM	DC	CC	BM	DC	CC	BM	DC
5	27.77	13.39	30.61	4.85	0	10.39	20.65	1.42	23.43
10	65.93	50.18	69.03	11.76	4.27	20.30	54.35	21.79	58.23
20	98.17	82.65	100.24	26.03	12.93	37.33	95.43	46.31	93.53
30	99.86	86.04	103.55	38.97	26.37	51.62	104.59	53.10	102.72
40	100.16	85.75	102.24	49.91	30.85	62.96	104.9	52.99	104.5
50	100.21	87.13	104.33	57.83	46.46	71.01	104.16	52.50	103.26
60	100.28	88.76	104.69	66.16	58.58	80.29	104.7	56.09	103.28

ture of 37°C, sampling times of 5, 10, 20, 30, 40, 50 and 60 min, and a sample volume of 5 ml, with the reposition, non-sink method [23,24].

The samples taken at respective times were filtered with nylon membrane of 0.45 μm pore, and then analyzed by HPLC (as already described).

Each active principle dissolved at each time, expressed as a percentage, is shown in Table 4.

4. Conclusion

The method to determine CC, BM and DC by HPLC described here has good linearity, accuracy and precision, and is simple and rapid to perform.

This method could be recommended for the control of CC, BM and DC contents in pharmaceutical formulations, as well as for the follow-up of samples obtained in a dissolution study.

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