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Validated high-performance liquid chromatographic methods for quantitation of a novel nonsteroidal antiestrogen

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Abstract

HPLC assays were developed and validated for the quantitation of the novel orally active nonsteroidal antiestrogen EM-800 {(*S*)-(+)-4-[7-(2,2-dimethyl-1-oxopropoxy)-4-methyl-2-[4-[2-(1-piperidinyl)-ethoxy]phenyl]-2H-1-benzopyran-3-yl]-phenyl 2,2-dimethylpropanoate}. The assay involves reversed-phase C_{18} or C_4 columns using different mobile phases with ammonium acetate buffers and UV detection at λ=240 nm. The standard curve was linear over the concentration range of 10–1100 μg/ml. The precision (% relative standard deviation) values of these methods were in the range of 0.38–0.52 and 1.89–3.45% with C_4 and C_{18} reversed phases, respectively. The limit of detection was found to be 1 μg/ml. Enantiomeric separation was also obtained using a chiral method (ChiralPak AD column) using a mixture of hexane–reagent alcohol–diethylamine (94.9:5.0:0.1) as mobile phase. These methods were applied to stability studies, evaluation of pharmaceutical dosage forms and in the framework of toxicological studies. Details of some of these applications will be presented.

Keywords: Validation; Enantiomer separation; Pharmaceutical analysis; Antiestrogens; EM-800

1. Introduction

Breast cancer is the most frequent cancer and the second cause of cancer death in women in North America [1]. Since estrogens play a predominant role in the development and growth of human breast cancer, antiestrogens represent a logical approach for the treatment of this disease [2–8]. Unfortunately, until very recently, no compound having pure antiestrogenic activity in breast tissue has been available. In fact, Tamoxifen, the only antiestrogen widely available for the treatment of breast cancer in women, behaves as a mixed agonist/antagonist of estrogen action, thus limiting its therapeutic potential [9–11]. We have thus developed a novel orally active nonsteroidal antiestrogen, EM-800 {(*S*)-(+)-4-[7-(2,2-dimethyl-1-oxopropoxy)-4-methyl-2-[4-[2-(1-piperidinyl)-ethoxy]phenyl]-2H-1-benzopyran-3-yl]-phenyl 2,2-dimethylpropanoate} [12]. As a crucial part of the drug development process, validation of analytical procedures is a required process for determining the suitability of a given methodology for providing the validated analytical data [13]. Therefore, different analytical methods were developed and validated in order to assay the compound EM-800 in non-clinical, clinical and toxicology studies. Different HPLC methods using reversed-phases were developed to assay the drug substance and another one was developed specifically to obtain the chiral separation of the racemic drug substance. In the latter case, the enantiomers (the (−)-enantiomer EM-776 and the (+)-enantiomer EM-800) were sepa-

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rated with a chiral stationary phase. The assay validations were first focused on the drug substance for support of stability studies. An assay was also extended to pharmaceutical dosage forms to support the quality control and stability studies. This assay has also been used, with slight modifications, as support of toxicology studies to determine the concentration of the drug substance in animal feed.

2. Experimental

2.1. Chemical and reagents

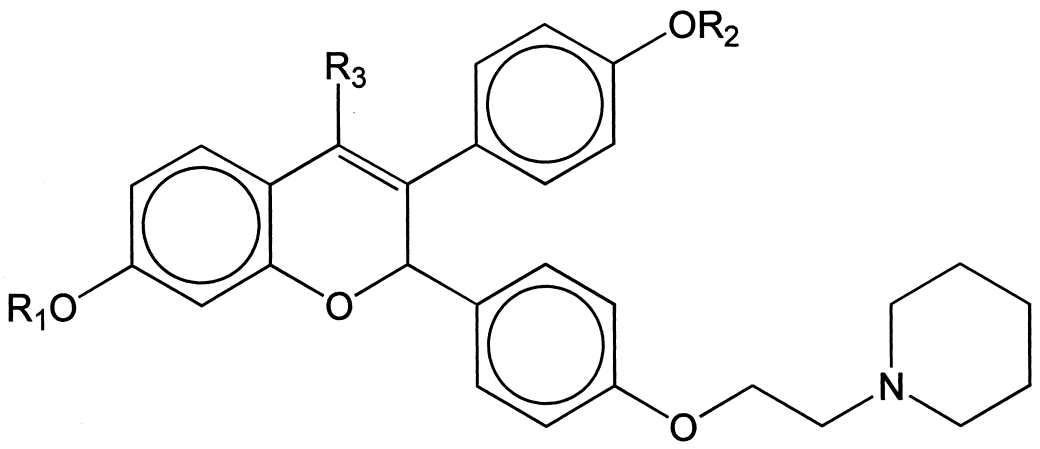
The compounds EM-652, EM-776, EM-800, EM-831 and EM-832 were synthesized in the Medicinal Chemistry Division of our laboratory (Fig. 1). HPLC-grade methanol, acetonitrile, chloroform, hexane and reagent alcohol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium acetate was purchased from Aldrich (Milwaukee, WI, USA), while diethylamine (99.5% purity) was obtained from Sigma (St. Louis, MO, USA). The water used in mobile phases was freshly distilled, deionized and purified in a Milli-Q equipment (Millipore, Bedford, MA, USA). All solvents were filtered through a 0.45-μm membrane in a Millipore filtration system before chromatographic analysis.

2.2. Apparatus and chromatographic conditions

Analyses were performed using a Waters Model 600E System Controller, a Waters Model 717 Autosampler and Waters Models 991 and 996 photodiode array detectors (Waters, Milford, MA, USA). All calculations were performed using Waters 991M software and Waters Millennium 2010 PDA software.

Four HPLC methods were used: System I for the analysis of the drug substance alone or in capsules, System II for the analysis of the drug substance, System III for the analysis of the drug substance in animal feed and System IV for the enantiomeric separation.

System I used a YMC-Pack C_4 column (250×4.6 mm I.D., 5 μm, YMC, Wilmington, NC, USA) with



EM-652 :	R_1=H	R_2=H	R_3=CH_3	(+) enantiomer
EM-800 :	R_1=$COC(CH_3)_3$	R_2=$COC(CH_3)_3$	R_3=CH_3	(+) enantiomer
EM-776 :	R_1=$COC(CH_3)_3$	R_2=$COC(CH_3)_3$	R_3=CH_3	(-) enantiomer
EM-832 :	R_1=H	R_2=$COC(CH_3)_3$	R_3=CH_3	racemic
	R_1=$COC(CH_3)_3$	R_2=H	R_3=CH_3	
EM-831 :	R_1=$COC(CH_3)_3$	R_2=$COC(CH_3)_3$	R_3=H	racemic

Fig. 1. Chemical structures of EM-800 and related compounds.

an isocratic component and two linear gradients. Solvent A was water containing 10 mmol/l ammonium acetate and solvent B was methanol containing 10 mmol/l ammonium acetate. The conditions were isocratic with 20% A and 80% B for 20 min. A gradient ran from this composition to 100% B in 5 min, this condition being held for 5 min before a second gradient ran to initial conditions in 5 min. The column was then re-equilibrated to the initial conditions for 10 min. The first gradient was used to eluate all impurities present in the samples and the second gradient was used to obtain the initial conditions. The photodiode array was set at 240 nm. The compound of interest EM-800 elutes in the isocratic part, but the degradation products of EM-800 are not well separated using this method. System I was used to assay EM-800 in stability studies and in pharmaceutical dosage forms.

System II employed a Waters Nova-Pak C_{18} (150×3.9 mm I.D., 4 μm). The method consisted of two linear gradients and an isocratic component. Solvent A was methanol–water (1:1, v/v)–10 mmol/l ammonium acetate, while solvent B was methanol containing 10 mmol/l ammonium acetate. The gradient began with 100% A and went to 100% B in 20 min. This composition was held for 5 min and a second gradient ran from this composition to 100% A in 5 min. The column was then re-equilibrated to the initial conditions for 15 min. The photodiode array was set at 240 nm. This method was developed to have a better separation of the degradation products, and it was used as a second method for the determination of degradation products in stability studies. Because the compound of interest was eluted in the gradient, this method was less precise and it was not chosen to assay EM-800 in pharmaceutical dosage forms.

System III used chromatographic conditions that were identical to those of System I, except that UV absorbance was at 320 nm and the conditions were isocratic with a mobile phase composed of water containing 10 mmol/l ammonium acetate–methanol containing 10 mmol/l ammonium acetate (20:80) during 30 min. This method was developed to assay the compound EM-800 in animal feed. Animal diet is a very complex matrix containing a great number of compounds as lipids, proteins, fibers, pesticides and it was not possible to use System I because of interferences between EM-800 and other compounds at 240 nm. As EM-800 shows different wavelengths maxima (240 and 320 nm) and no interference was observed at 320 nm, this method was used to assay EM-800 in animal feed. The method III was not used in stability studies of the drug substance because the absorbance is twice as great at 240 nm and it is more judicious to have high sensibility in stability studies.

For the enantiomeric separation in System IV, the column used was a Daicel ChiralPak AD column (250×4.6 mm I.D., 10 μm) purchased from Chiral Technologies, Exton, PA, USA. The mobile phase was a mixture of hexane–reagent alcohol–diethylamine (94.9:5.0:0.1). The run time was 20 min and the detection was UV absorbance at 240 nm.

In all these systems, the column temperature was held at 25±2°C and the flow-rate was 1 ml/min.

2.3. Standard solutions and preparation of samples

All standard solutions were prepared daily in methanol–chloroform (1:1) in the case of Systems I and II because the drug substance EM-800 is fairly soluble in alcohol. For determination of the concentration of EM-800 in animal diet, stock solutions of EM-831 were prepared in acetonitrile. In case of the enantioselective separation with System IV, a mixture of hexane–chloroform (9:1) was used as reference solvent for standard solutions.

For the determination of EM-800 in capsules, the procedure was the following: a composite assay was obtained by extraction of the drug substance from 10 capsules placed together in the appropriate volume of solvent. The contents and shells of 10 capsules were placed in a flask containing methanol–chloroform (1:1) of appropriate volume (depending of the drug substance content) and the capsule contents were dissolved using an ultrasonic bath for 10 min. The mixture was then shaken vigorously for 10 min with a mechanical vortex. After decantation for at least 45 min, six aliquots of supernatant were taken, filtered on a 0.45-μm membrane and assayed versus an external standard prepared in methanol–chloroform (1:1). The content uniformity was analysed on 10 individual capsules having the same preparation as the composite assay.

The next method to be described is the one for determination of the concentration of EM-800 in

animal diet preparations containing between 0.313 and 20.0 mg EM-800/g of feed. Quantification was achieved using the internal standard method (EM-831 as internal standard). Samples (2.4 g) of EM-800-containing feed were extracted with acetonitrile containing EM-831 as internal standard. The ratio of the EM-800 peak area to the EM-831 peak area was calculated and the concentration of EM-800 in feed was determined by comparing the EM-800/EM-831 ratio obtained from separately prepared reference standards extracted and analysed in the same way as samples.

Solid-phase extraction cartridges (Sep-Pak Vac C_{18}, 6 ml, 1 g) were obtained from Waters. For the study of robustness, cartridges from Supelco were also used (Supelclean, LC-18, 6 ml, 1 g). One extraction cartridge was used per sample and it was discarded after use. The sample preparation was the following: the solid-phase extraction column was activated by washing with 2 ml of methanol and 5 ml of water. A 1-ml volume of reference standard solution or sample was extracted. The column was washed with 3 ml of acetonitrile–water (1:1) and 1 ml of methanol. The drug substance was finally eluted with 10 ml of methanol in a class A 10.0-ml volumetric flask. Following this procedure, a 20-μl aliquot was injected for the 1.25-, 10.0- and 20.0-mg EM-800/g feed doses, while an 80-μl aliquot was injected for the 0.313-mg EM-800/g feed dose.

To ensure the efficiency of the extraction procedure, separately prepared control samples analogous to the reference standard samples were injected into the HPLC. The control was extracted after the samples and analysed in comparison with the reference standard. The recovery of the analyte was determined by comparing the calculated concentration with its theoretical concentration.

2.4. Analytical and validation procedures

The following tests were performed to assay the drug substance with the HPLC Systems I and II: specificity, linearity, limit of detection, limit of quantification, precision as well as robustness. Specificity indicates the ability of an analytical method to measure the analyte to the exclusion of relevant components which might interfere (synthesis-related compounds, degradation products). Assays were performed after stressing the drug substance to accelerate degradation under the influence of acid and base hydrolysis, oxidation, heat and in solution. Linearity of the response was tested in the concentration range between 2 and 180% of the target concentration (0.6 mg/ml). The limit of detection was determined for a signal-to-noise ratio of 3:1, and the limit of quantitation was defined as the lowest concentration of the drug substance which can be quantified reliably with an acceptable level of accuracy and precision. Precision was performed by analysis of 10 replicate injections of the analyte at the target concentration on two different days. Robustness was studied by variations in method parameters as mobile phase changes (variation in the procedure mobile phase ratio), column temperature (variation from 23 to 27°C) and sample solution stability for at least 9 h.

In order to assay the drug substance in pharmaceutical dosage forms as capsules with the HPLC System I, the following tests were performed: placebo interference, linearity, accuracy, composite assay and content uniformity. Placebo interference was evaluated by examination of chromatograms from placebo capsules and the drug substance. In order to demonstrate linearity and accuracy of the assay with capsules, the following experiments were performed: a series of samples were prepared by simulating the capsule formulation process by the addition of different amounts of the drug substance to the placebo in concentrations ranging from 50 to 175% of the label. Recovery was further determined by the difference between the nominal and the measured concentrations as part of determination of the accuracy of the assay. Composite assay and content uniformity were then performed as described above.

For the determination of the drug substance in animal feed with HPLC System III, the following tests were performed: extraction efficiency, matrix interference, precision, accuracy, linearity and robustness. The extraction efficiency was determined using 0.313 and 20.0 mg drug substance/g feed. Three samples of each concentration were extracted with appropriate internal working solution as previously described, but with varying sonication time intervals. The durations of sonication were 10, 20 or 30 min. Concentrations of drug substance in feed

were compared to standard solutions (0.015 and 0.12 mg drug substance/ml for 0.313 and 20.0 mg drug substance/g feed, respectively). Extraction efficiency needed to be at least 80% for the sonication time intervals used.

To verify the non-interference of the matrix (feed) on EM-800 and EM-831 determinations, blank samples prepared by adding 5 ml of acetonitrile to 2.4 g of feed (matrix) were used. After extraction, aliquots were injected in duplicate into the HPLC system, and then the chromatogram of a blank sample was compared to a chromatogram of the standard solution to prove that matrix did not interfere with EM-800 and EM-831 determination.

To determine the precision of the method, control samples were prepared at 0.313, 1.25, 10.0 and 20.0 mg EM-800/g feed on two different days. Six extractions were performed on each of the control samples and compared to the corresponding standard sample concentrations. Two extractions were performed on standard samples, before and after extraction of the control samples. To determine the linearity of the method, different investigations were conducted on extracts of feed having different concentrations of EM-800: duplicate preparations at 80, 100 and 120% of each dose were diluted in 5 ml of the internal standard working solution (0.0625 mg EM-831/ml) and extracted as previously described for injection in duplicate into the HPLC system. The ratio of EM-800/EM-831 area was plotted against the theoretical concentration of EM-800 in the solution prior to extraction.

The following parameters were tested to verify the robustness of the method: extraction by two analysts to prove that results obtained by the method were consistent independently of involved technical personnel, stability of solutions obtained after extraction and extraction using different cartridges.

For the determination of the enantiomeric purity of the drug substance with System IV, the following tests were performed: linearity, precision and robustness. Linearity was studied with each of the enantiomers and with different solutions of EM-776 spiked with a known amount of EM-800 to observe the effects of this compound on the EM-776 response. Precision was performed by analysis of 10 replicate injections of a solution containing 98% EM-800 and 2% EM-776 on two different days. Robustness was studied by small variations in method parameters as mobile phase changes (variation in the procedure mobile phase ratio), column temperature (variation from 25 to 35°C), flow-rate (variation from 0.5 to 1.5 ml/min) and sample solution stability at ambient temperature for at least 12 h.

3. Results and discussion

3.1. Analysis of the drug substance with HPLC Systems I and II

The hydrolysis of EM-800 into the dihydroxy-free active antiestrogen EM-652 is observed in the blood. EM-832 is a mixture of monopivalates which are intermediates in this transformation of EM-800 into EM-652. Typical chromatograms obtained with methods I and II are shown in Fig. 2. As can be seen, EM-832 is well separated from EM-800 with the two systems, but the resolution between the two monopivalates is better with method II. No interfering peaks were found at the retention time of EM-800 in the study of specificity. EM-800 was well separated from possible decomposition products.

The results for linearity and precision are summarized in Table 1. The response was linear and unbiased and the methods showed enough precision to be used to adequately determine the drug substance. Under the conditions of the assay, the limit of detection was 1 μg/ml and the lower limit of quantitation (LOQ) was 10 μg/ml.

Resolution between the drug substance EM-800 and some of the known related products (EM-652, EM-832) was observed under different mobile phase compositions used to study the robustness of the methods. These results are presented in Tables 2 and 3. Longer retention time and improved separation was achieved by reducing the methanol concentration. Conversely, the retentions was shortened with some loss of resolution by increasing the methanol concentration. These data demonstrate that the mobile phase ratio is a critical parameter which influence the elution profile of the related compounds of EM-800 and EM-800 itself. A significant change of pH of the aqueous mobile phase (pH adjusted to 3.0) affected the separation with a decrease in the resolution achieved. A slight variation of column

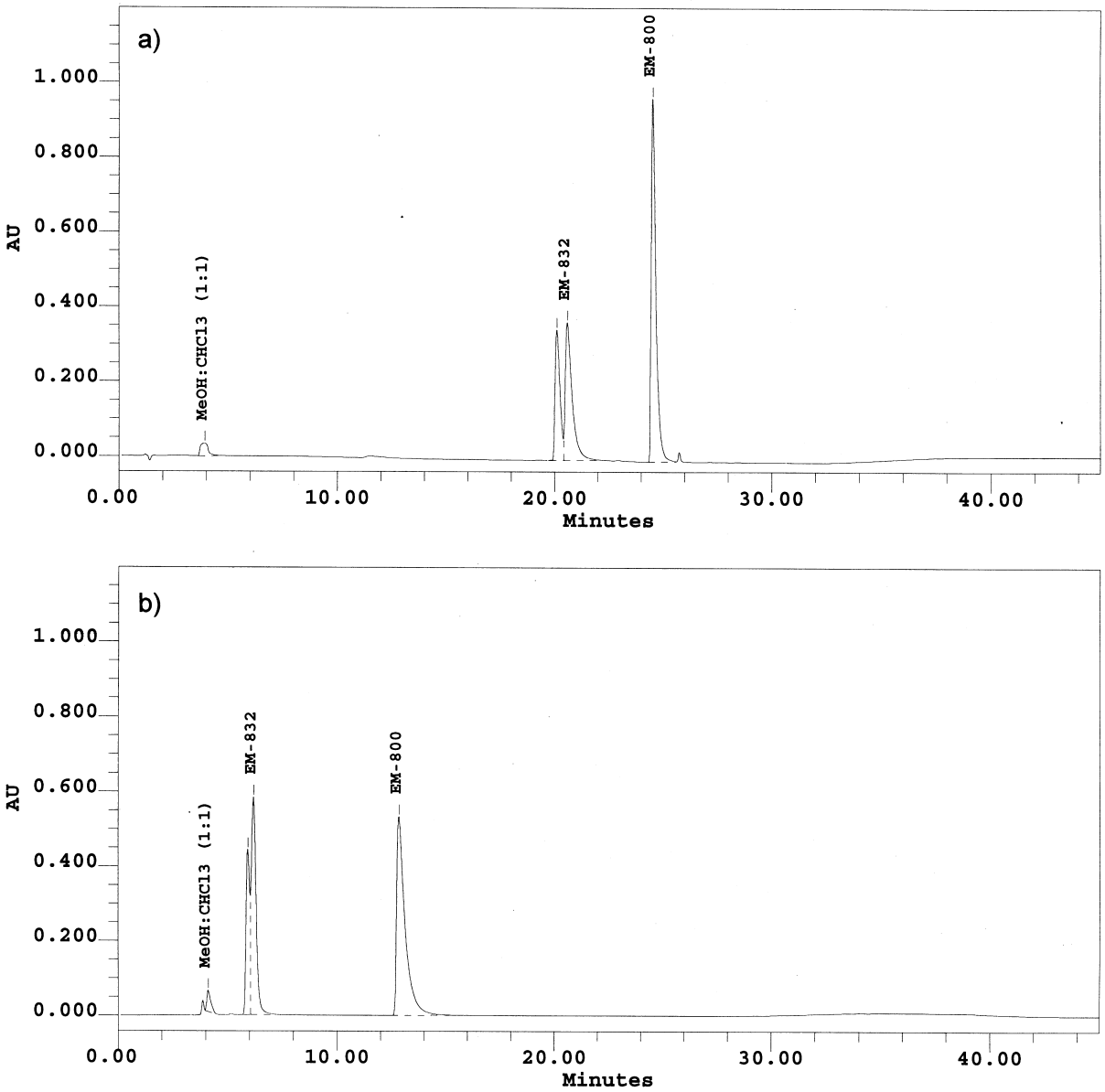


Fig. 2. Typical chromatograms of a solution containing EM-832 and EM-800 at a 0.3-mg/ml concentration with System II (a) and System I (b). Chromatographic conditions are described in the text.

temperature did not change the results. The stability of a sample solution was investigated by performing repeated assays at 3 h intervals up to 9 h. No degradation was detected under the experimental conditions used and relative assay value in comparison with initial time of measure was 102%.

3.2. Analysis of the drug substance in capsules with HPLC System I

Fig. 3 shows a typical chromatogram obtained from a standard and a placebo capsule. The superimposable overlay shows no significant interferences by

Table 1
Retention time, linearity and precision

System	Retention time (min)[a]	Linear regression equation, coefficient of determination	Precision ($n=10$)
I[b]	13.74±0.03	$y=0.00086x+0.0047r^2=0.9998$	Day 1: R.S.D.=0.52%Day 2: R.S.D.=0.38%
II[b]	24.51±0.02	$y=4.72\times10^{+4}x+8.06\times10^{+5}r^2=0.9935$	Day 1: R.S.D.=1.89%Day 2: R.S.D.=3.45%

Linear regression equation $y=Ax+B$, with A the regression coefficient, B the intercept, y the peak area of EM-800 and x the concentration of EM-800.
[a]From precision data of day 1.
[b]System I, data obtained with Waters 991M software; System II, data obtained with Waters Millennium 2010 PDA sofware.

Table 2
Robustness: retention time (min) of different compounds according to variation of mobile phase compositions

System I	A–B (20:80) pH (A) 7.00 (initial conditions)	A–B (15:85)	A–B (25:75)	A–B (20:80) pH (A) adjusted to 3.01
	$t_{R1}=3.96$ $t_{R2}=6.19, 6.40$ $t_{R3}=12.98$	$t_{R1}=3.91$ $t_{R2}=5.31$ $t_{R3}=8.65$	$t_{R1}=4.25$ $t_{R2}=8.61, 9.05$ $t_{R3}=25.52$	$t_{R1}=3.94$ $t_{R2}=6.08, 6.27$ $t_{R3}=12.35$
System II[a]	A: MeOH–water (50:50) pH (A) 6.49 (initial conditions)	A:MeOH–water (40:60)	A:MeOH–water (60:40)	A:MeOH–water (50:50) pH(A) adjusted to 3.00
	$t_{R3}=24.30$	$t_{R3}=25.23$	$t_{R3}=23.23$	$t_{R3}=21.16$

t_{R1}, retention time of EM-652; t_{R2}, retention time of EM-832; t_{R3}, retention time of EM-800.
[a]All A eluents contain 10 mmol/l ammonium acetate.

the placebo components with the drug substance recovery. The results of different tests are presented in Table 4. The linearity of the method was demonstrated over 50–175% of the procedure concentration. The coefficients of determination were within the range 0.9992–0.9999. The recovery obtained from linearity data was calculated at a value ranging between 98.9 and 99.8% with a R.S.D. inferior or equal to 1.77%, thus showing the accuracy of the method. The results of the composite assay range between 94.0 and 97.7%, depending upon the formulation. The requirements for uniformity content were met since the amount of the active ingredient in each of the 10 dosage units was within the range 85–115% of the label claim. In fact, the values obtained were between 94.1 and 101.1%, with a R.S.D. value

Table 3
Robustness: retention time (min) of different compounds according to variations of column temperature

	$T=25$°C (initial conditions)	$T=23$°C	$T=27$°C
System I	$t_{R1}=3.99$ $t_{R2}=6.12, 6.32$ $t_{R3}=12.23$	$t_{R1}=3.98$ $t_{R2}=5.97, 6.15$ $t_{R3}=11.48$	$t_{R1}=3.99$ $t_{R2}=6.09, 6.28$ $t_{R3}=12.11$
System II	$t_{R1}=10.57$ $t_{R2}=20.00, 20.42$ $t_{R3}=24.30$	$t_{R1}=10.95$ $t_{R2}=20.10, 20.51$ $t_{R3}=24.36$	$t_{R1}=10.38$ $t_{R2}=19.90, 20.32$ $t_{R3}=24.23$

t_{R1}, retention time of EM-652; t_{R2}, retention time of EM-832; t_{R3}, retention time of EM-800.

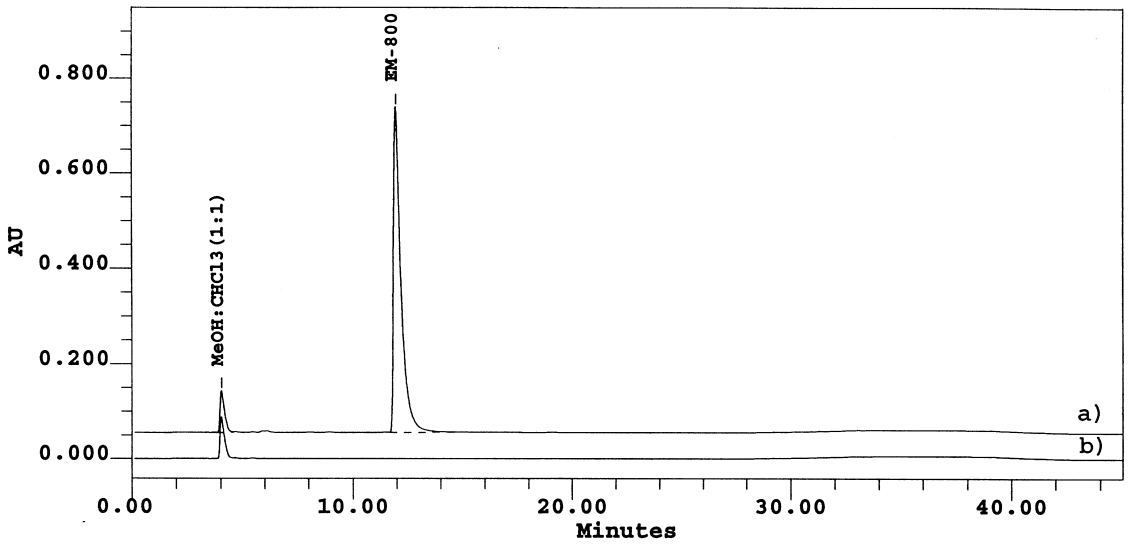


Fig. 3. Chromatograms of a 0.33-mg/ml EM-800 solution (a) and a placebo capsule (b) obtained with System I. Chromatographic conditions are described in the text.

between 2.03 and 4.79%, depending upon the formulation.

3.3. Analysis of the drug substance in animal feed

The results for extraction efficiency are shown in Table 5. Extraction proved to be sufficient at values ranging between 86.0 and 99.6% of extraction efficiency for diets containing 0.313 and 20 mg EM-800/g, respectively. The sonication time retained for further tests was 20 min. Representative chromatograms of diet and diet containing EM-800 are presented in Fig. 4. No interferent peak was found at a retention time of EM-800.

The different tests of precision, linearity and accuracy are presented in Table 6. The R.S.D. value for intra-day precision was less or equal to 1.24%. The test results indicate that the method shows an adequate linearity: the coefficient of determination r^2 in all cases was greater than or equal to 0.9957. Recovery in terms of accuracy was obtained from individual calculated concentrations from linearity data and ranged from 99.9 to 100.2% with a R.S.D. value ranging from 0.21 to 0.91%.

Table 7 shows the results obtained by two

Table 4
Analysis of EM-800 in capsules

	Linearity parameters curve, r^2	Accuracy[a]: recovery (%), R.S.D. (%)	Composite assay: recovery (%)	Content uniformity: recovery (%), R.S.D. (%)
1.0-mg capsules	$y=51716021x-26615$	99.8	94.0	94.1
	0.9999	0.58		2.03
2.5-mg capsules	$y=53135424x-101363$	99.8	96.0	94.8
	0.9992	1.77		2.27
5.0-mg capsules	$y=52927149x-126250$	99.6	97.7	98.7
	0.9999	0.69		3.98
10.0-mg capsules	$y=52269615x-239257$	98.9	97.6	101.1
	0.9995	1.37		4.79

[a]From linearity data.

Table 5
Extraction efficiency for the determination of EM-800 in feed

Amount of EM-800 in diet (mg EM-800/g diet)	Sonication time (min)	Recovery (%)
0.313	10	86.0
	20	86.1
	30	87.3
20.0	10	99.4
	20	99.6
	30	99.4

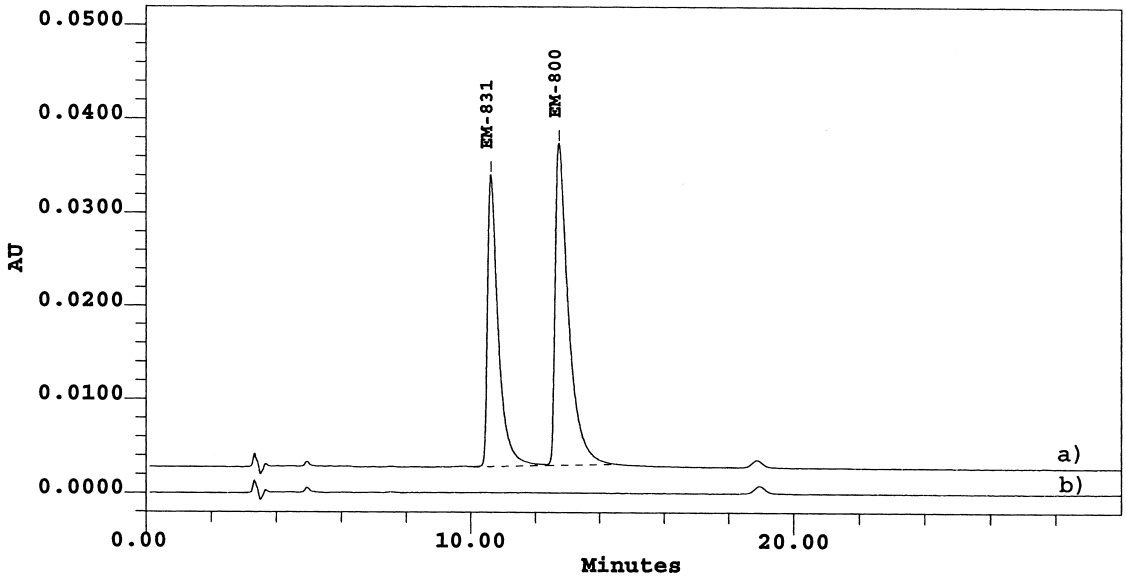


Fig. 4. Chromatograms of a diet containing EM-800 at 1.25 mg/g (a) and diet alone (b) with HPLC System III. Chromatographic conditions are described in the text.

Table 6
Precision, linearity and accuracy for the determination of EM-800 in feed

Amount of EM-800 in diet (mg EM-800/g diet)	Precision ($n=6$) R.S.D.	Linearity parameters curve, r^2	Accuracy: recovery (%) (R.S.D.)
0.313	Day 1: 0.81%	$y=115.15x-0.26$	100.2
	Day 2: 1.24%	0.9957	(0.91%)
1.25	Day 1: 0.34%	$y=27.88x-0.14$	100.0
	Day 2: 1.11%	0.9995	(0.38%)
10.0	Day 1: 0.31%	$y=27.71x-0.12$	100.0
	Day 2: 0.44%	0.9998	(0.21%)
20.0	Day 1: 0.11%	$y=26.31x-0.01$	99.9
	Day 2: 0.20%	0.9996	(0.22%)

Linear regression equation $y=Ax+B$, with A the regression coefficient, B the intercept, y the ratio EM-800/EM-831 and x the theoretical concentration of EM-800.

Table 7
Precision test by two analysts using diet containing 10 mg EM-800/g

Analyst	Nominal concentration (mg/ml)	Measured concentration (mg/ml)	Recovery (%)
1	0.1200	0.1203	100.3
	0.1200	0.1199	99.9
	0.1200	0.1208	100.7
	0.1200	0.1206	100.5
	0.1200	0.1208	100.7
	0.1200	0.1207	100.6
			Mean=100.5%
			R.S.D.=0.31%
2	0.1193	0.1194	100.1
	0.1193	0.1190	99.7
	0.1193	0.1191	99.8
	0.1193	0.1196	100.3
	0.1193	0.1193	100.0
	0.1193	0.1196	100.3
			Mean=100.0%
			R.S.D.=0.25%

analysts for the extraction of 10 mg EM-800/g feed ($n=6$). The recovery indicated a R.S.D. value less or equal to 0.31%.

Sample solutions obtained following extraction were tested at room temperature and at 4°C and they showed no change for at least 96 h. Relative assay in comparison with initial time ranged between 100.1 and 100.8%.

The results obtained with two different extraction cartridges were the following: the recovery for Waters cartridges was 99.7% with a R.S.D. equal to 0.81% and the recovery for Supelco cartridges was 98.7% with a R.S.D. equal to 3.28%. Consequently, Waters cartridges were used for the following studies.

3.4. Enantioselective separation

As can be seen in Table 8, linearity was studied under different conditions. In all cases, the response was linear and unbiased and the coefficient of determination was superior or equal to 0.9988. The linearity parameters of EM-776 spiked with a known amount of EM-800 were slightly affected by the presence of EM-800. A typical chromatogram of a solution containing 98% of EM-800 and 2% of EM-776 is shown in Fig. 5.

The results of precision are presented in Table 9. The R.S.D. value was in all cases inferior or equal to 1.44% for the retention time as well as the percent of peak area. Resolution between the two compounds was always superior to 1.50, thus indicating good separation between EM-800 and EM-776.

The results of robustness tests are presented in Tables 10 and 11. A decrease of the percentage of hexane in the mobile phase decreased the retention time of EM-800 and EM-776 and also the resolution. Conversely, longer retention time and improved separation was achieved by increasing the percentage of hexane. These data show that it is important to have a minimum of 94.9% hexane in the mobile phase. Moreover, as can be seen in Table 11, with an

Table 8
Linearity parameters obtained with the enantioselective chiral column

Parameter	EM-800	EM-776	EM-776 spiked with 40 μl of a 25-mg/ml EM-800 solution
Concentration range	0–140 mg/ml	0–0.05 mg/ml	0–0.05 mg/ml
Curve	$y=45925946x+698022$	$y=47694329x-30752$	$y=48736276x-16484$
r^2	0.9988	0.9994	0.9998

Linear regression equation $y=Ax+B$, with A the regression coefficient, B the intercept, y the peak area response and x the concentration of EM-800 or EM-776.

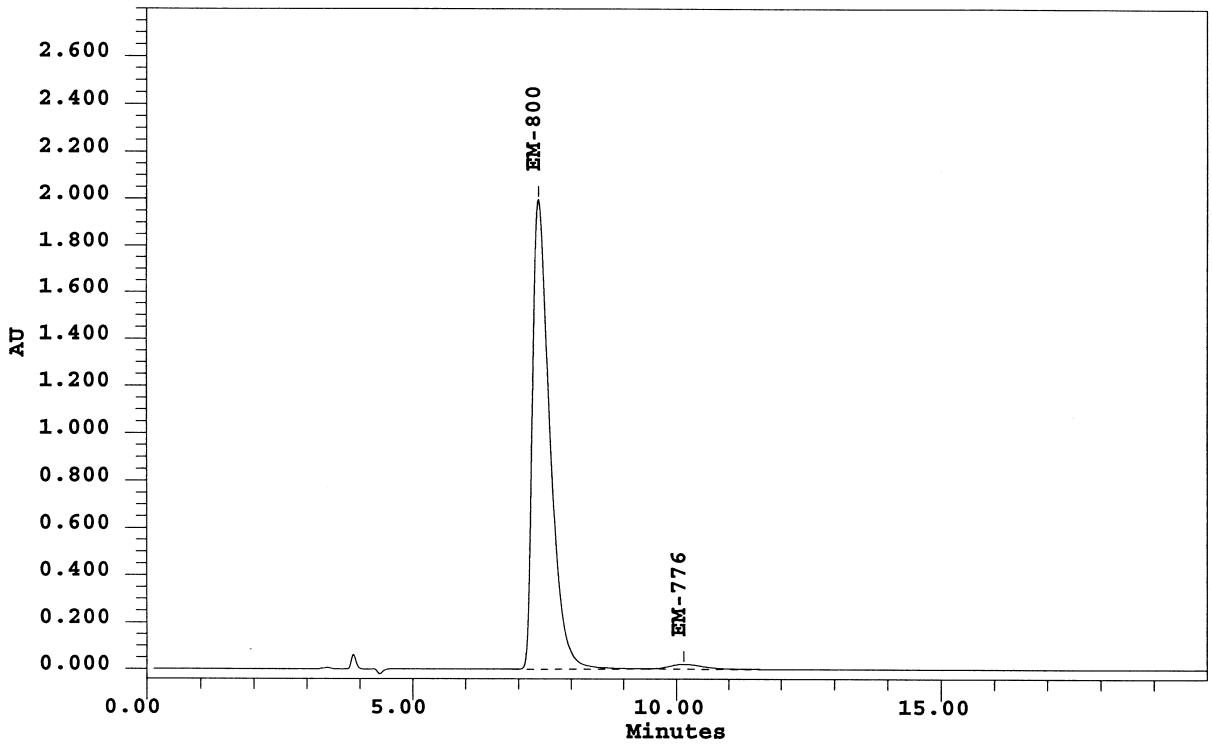


Fig. 5. Chromatograms of a solution containing 98% EM-800 and 2% EM-776 with the enantioselective column (System IV). Chromatographic conditions are described in the text.

Table 9
Retention time, percent peak area and resolution with precision using the chiral column

	Retention time (min)		% Peak area		Resolution	
	Mean±S.D.	R.S.D. (%)	Mean±S.D.	R.S.D. (%)	Mean±S.D.	R.S.D. (%)
Day 1 (n=10)						
EM-800	7.98±0.05	0.63	98.05±0.02	0.02	1.68±0.03	1.75
EM-776	11.48±0.17	1.44	1.95±0.02	0.95		
Day 2 (n=10)						
EM-800	8.06±0.02	0.30	98.00±0.01	0.01	1.84±0.03	1.77
EM-776	11.73±0.08	0.67	2.00±0.01	0.44		

Table 10
Retention time (min) and resolution according to variation of mobile phase composition using the chiral column

	Hexane–ethanol–diethylamine (94.9:5.0:0.1) (initial conditions)	Hexane–ethanol–diethylamine (84.9:15:0.1)	Hexane–ethanol–diethylamine (89.9:10:0.1)	Hexane–ethanol–diethylamine (97.9:2.0:0.1)
EM-800	8.69	5.54	6.34	14.98
EM-776	13.26	6.99	8.48	27.48
Resolution	1.84	1.01	1.05	2.96

Table 11
t_R (min) and resolution according to variation of flow-rate and column temperature using the chiral column

	Flow-rate (ml/min)			Column temperature (°C)		
	0.5	1.0	1.5	25	30	35
EM-800	16.62	8.13	5.51	8.36	7.94	7.40
EM-776	24.21	11.86	8.05	12.23	11.52	10.67
Resolution	2.38	1.48	1.04	1.56	1.57	1.61

increasing flow-rate, the retention time as well as the resolution decreased. An increase in column temperature gave a shorter retention time but the resolution was only slightly affected. The stability of a sample racemic solution containing 50% EM-800 and 50% EM-776 was investigated by performing repeated injections at 3-h intervals up to 12 h at ambient temperature. No degradation was observed under the experimental conditions used (same retention time, percent peak area and resolution) indicating the stability of the racemic solution up to 12 h at ambient room temperature.

4. Conclusion

The present chromatographic methods developed for the analysis of the drug substance EM-800 are linear, precise, reproducible and sufficiently robust. The methods were suitable for the measurement of EM-800 concentrations in a variety of different matrix as pharmaceutical formulations (capsules) and animal feed.

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