Separation from Related Compounds and Assay of Calcipotriol by High-Performance Liquid Chromatography

Jesusa Joyce N. Cirunay[1], Yvan Vander Heyden[2], and Jacqueline Plaizier-Vercammen[1,*]
[1]Department of Pharmaceutical Technology and Physical Pharmacy and [2]Department of Pharmaceutical and Biomedical Analysis, Pharmaceutical Institute, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090, Brussels, Belgium

Abstract

In this paper, two methods are presented. One involves the separation of calcipotriol, a new synthetic analogue, from two related compounds, specifically cholecalciferol (Vitamin D_3) and calcitriol (1,25-dihydroxyvitamin D_3). The other involves the isolation and assay of calcipotriol from a topical ointment. The study was performed with reversed-phase high-performance liquid chromatography using an RP18 column and ultraviolet detection. Applying the method of Snyder, a mobile phase mixture containing methanol–acetonitrile–water (67:23:10, v/v) was found which achieved a total separation within 18 min. A mobile phase of methanol–water (80:20, v/v) attained a slower elution of calcipotriol. For isolation and assay of calcipotriol from an ointment (Daivonex), dissolution in chloroform gave the highest recovery (> 98%). The isolation and assay process can be performed within 2 h.

Introduction

Since the identification of the anti-ricketic factor as Vitamin D in 1923, studies on this substance have increased in number (1). At present, Vitamin D is known for its regulation of calcium absorption, bone mineralization, and prevention of rickets (2). However, after the coincidental discovery by Japanese researchers of the effect of a Vitamin D_3 product on the improvement of a patient's psoriasis while being treated for osteoporosis, research has been focused on exploring Vitamin D_3 analogues for the treatment of psoriasis, a disease characterized by hyperproliferation and incomplete differentiation of the epidermis (3).

In recent years, a new synthetically prepared analogue called calcipotriol has been identified as a topical antipsoriatic agent (3–5). It can regulate cell differentiation and proliferation in vitro and suppress lymphocyte activities. These actions of calcipotriol at the cellular level are very similar to calcitriol (1,25-dihydroxyvitamin D_3, a natural hormone), yet the influence on calcium and bone metabolism is far less (6,7). Calcitriol is the most active metabolic product of Vitamin D_3, a photochemical product of the skin formed after exposure of 7-dehydrocholecalciferol to ultraviolet (UV) sunlight. However, there is no method described for the separation of these three substances in a mixture. Individual determinations of calcipotriol (8), calcitriol (9), and cholecalciferol (10,11) are available.

In this study, a separation procedure was developed using reversed-phase high-performance liquid chromatography (RP-HPLC). The mobile phase composition was optimized by applying the Snyder solvent triangle (12) for a mixture containing calcipotriol, calcitriol, and cholecalciferol. The separation procedure served as an additional control for the presence of related substances in the testing of raw material for any of the three Vitamin D_3 compounds. An isolation method for calcipotriol in a commercially available ointment (Daivonex) (5,13) was explored.

Experimental

Materials

Equipment

RP-HPLC analysis was performed on a LiChroCART 5-µm RP18 4 × 125-mm column (Merck, Darmstadt, Germany), a Merck-Hitachi L-6000A pump with a Perkin-Elmer LC 90 UV spectrophotometric detector (Norwalk, CT), and a Merck-Hitachi D-2500 integrator. The flow rate was 2 mL/min for separation and 1 mL/min for assay and recovery. The UV detection wavelength was 254 nm for separation and 263 nm for assay and recovery. The loop volume was 20 µL. A GFL Schüttelwasserbad 1083 (GFL mbH, Burgwedel, Germany) at 120 vibrations per minute was used for shaking during the isolation of a mixture containing the ointment and a solvent. All centrifugations were performed using a Mistral 400 refrigerated centrifuge (MSE, West Sussex, England). Spectrophotometric analysis was performed with a Uvikon 860 spectrophotometer (Kontron Instruments AG, Zürich, Switzerland).

Chemicals

Samples of calcipotriol and calcitriol crystals were provided by Schering AG (Berlin, Germany), and calcipotriol in isopropanol

*Author to whom correspondence should be addressed.

Reproduction (photocopying) of editorial content of this journal is prohibited without publisher's permission.

was provided by Leo Pharmaceuticals (Ballerup, Denmark). Daivonex 50-µg/g ointment was manufactured by Leo Pharmaceuticals (Denmark). Cholecalciferol was purchased from Sigma-Aldrich (Bornem, Belgium). Chloroform and methanol were purchased from Merck. Acetonitrile for HPLC was purchased from CRB (Brussels, Belgium). Milli-Q water was purified using Seralpur Pro 90CN filters (Merck-Belgolabo, Overijse, Belgium). All solutions were prepared under red light at ambient temperature. Solutions were kept in amber-colored bottles wrapped in aluminum foil and stored at –5°C until use. Prior to and during HPLC analysis, samples were stored in a refrigerator at 8–15°C.

Methods and solutions

Separation and assay of calcipotriol

Stock solutions containing 100 µg/mL of the individual substances were prepared by dissolving the necessary amounts in methanol, which were then diluted further in methanol–water (80:20, v/v) to make 10-µg/mL solutions for injection.

Limit of detection and quantitation

A 100-µg/mL stock solution of calcipotriol was prepared by dissolving the necessary amounts in methanol. A working solution of 500 ng/mL and additional dilutions from 100 to 10 ng/mL were made in the mobile phase (methanol–water, 80:20, v/v).

Determination of calcipotriol in the ointment

One gram of the ointment containing 50 µg calcipotriol was dissolved in 10 mL chloroform, shaken for 1 h, then centrifuged at a relative centrifugal force of 1562 × g for 10 min at –5°C. One milliliter of the clear portion was pipetted out and diluted 2.5 times with methanol to make a theoretical concentration of 2 µg/mL. This solution was again centrifuged under the same conditions, after which 1 mL of the clear portion was pipetted out and diluted with the mobile phase (methanol–water, 80:20, v/v) to make theoretical concentrations of 500 ng/mL and 1 µg/mL.

Recovery

The recovery was computed as the peak area of calcipotriol from the ointment divided by the corresponding peak area of a reference solution (standard) containing calcipotriol at one of two possible concentrations (e.g., 500 ng/mL or 1 µg/mL) in chloroform.

Results and Discussion

Since there are two related compounds for the analogue of interest, the study began with a search for a mobile phase able to

Table I. Respective Retention Times and Capacity Factors of Cholecalciferol, Calcitriol, and Calcipotriol in Different Mobile Phases at a Flow Rate of 1 mL/min

			Retention time (min)			Capacity factor (k′)		
Mobile phase*	Mixture (v/v)	SST	Cholecalciferol	Calcitriol	Calcipotriol	Cholecalciferol	Calcitriol	Calcipotriol
MeOH	100	2.6	4.76	1.35	1.24	2.97	0.14	0.05
MeOH–H_2O	95:5	2.4	14.63	1.92	1.60	14.19	0.99	0.66
ACN–H_2O	77:23	2.4	> 60	3.59	2.38	> 62	2.80	1.53
MeOH–ACN–H_2O	76:16:8	2.4	23.43	2.32	1.82	22.67	1.34	0.83
MeOH–ACN–H_2O	67:23:10	2.4	35.20	2.76	2.05	34.92	1.81	1.09
MeOH–ACN–H_2O†	67:23:10	2.4	17.32	1.38	1.03	34.85	1.86	1.12

* Abbreviations: MeOH, methanol; ACN, acetonitrile; H_2O, water; SST, solvent strength.
† The flow rate was increased to 2 mL/min.

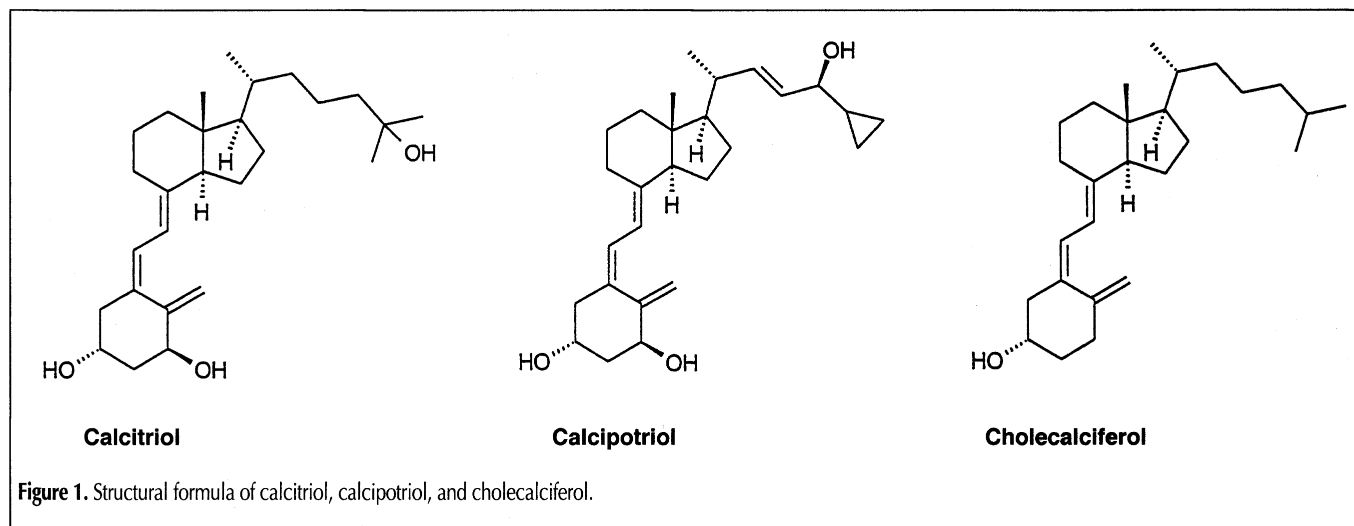

Figure 1. Structural formula of calcitriol, calcipotriol, and cholecalciferol.

separate calcipotriol from the related Vitamin D_3 compounds, cholecalciferol and calcitriol. This test for related substances is necessary because these three Vitamin D derivatives are closely related to each other (Figure 1). The varying levels of toxicity between the natural hormone calcitriol (metabolic byproduct of cholecalciferol) and the synthetically prepared Vitamin D_3 analogue calcipotriol are attributed to the fact that calcipotriol exhibits over 100 times less hypercalcemic effect (4,7) than calcitriol.

Separation of Calcipotriol from related compounds

Using Snyder's solvent classification scheme (12), a search for a good mobile phase composition was started, monitoring at 254 nm with an initial flow rate of 1 mL/min. Because detection at a fixed wavelength of 254 nm is commonly used, this wavelength was arbitrarily chosen in the beginning of our experiments to be the detection wavelength for the separation. The mobile phase optimization began with methanol as the selectivity solvent. Methanol was chosen because of previous data (unpublished) where the retention time of cholecalciferol was 4.5–4.7 min with pure methanol as mobile phase. However results showed that with pure methanol (solvent strength 2.6), undesirable capacity factor (k') values of 0.05 and 0.14 were obtained for calcipotriol and calcitriol, respectively, while cholecalciferol achieved a k' value of 2.97 (Table I). Several mobile phase mixtures were tried in an attempt to increase the capacity factors of calcipotriol and calcitriol while maintaining the solvent strength of the mixture. A mobile phase with two organic modifiers, methanol and acetonitrile, already gave better results but was not acceptable, because the k' of calcipotriol was less than 1. However, when the mobile phase composition was changed to methanol–acetonitrile–water (67:23:10, v/v) while maintaining the solvent strength at 2.4, the capacity factors for the three components all became larger than 1. The retention time of cholecalciferol was considered quite long at 35.20 min (k' = 34.92). The necessity of acquiring a k' value greater than 1 is explained in the following equation for the resolution of peaks (14):

$$Rs = \frac{1}{4}(\alpha - 1) \times (\sqrt{N}) \times \frac{k'}{1 + k'} \qquad \text{Eq 1}$$

where Rs is the resolution, a is the separation factor, N is the number of theoretical plates, and k' is the capacity factor. It is shown that Rs is proportional to $k'/(1 + k')$; therefore, increases in $k' > 10$ will have a very small effect on the resolution in comparison to those increases in the range of 1–10 (14). A k' range of 1–10 for all components of the sample is desirable but may be extended, depending upon separation complexity (15). By increasing the flow rate to 2 mL/min, a total running time of less than 18 min was achieved. The elution times of calcipotriol and calcitriol are relatively close, as can be noted but are still well separated from the injection peak, with k' = 1.12 and 1.86, respectively, and an Rs value of 1.27 between the analogues (Figure 2). It should be remembered that the aim of our experimental setup was to obtain a separation between calcipotriol and two of its related products, not necessarily to have a method able to quantitate all three substances. Therefore, we chose to use an isocratic method, if possible, with an acceptable run time. We considered the final results presented in Figure 2 acceptable for that purpose, and for that reason no further attempts were made to optimize a gradient method. Also, it was feared that the analysis time of such a method would not be shorter, taking into account both the run time and the time to reequilibrate the column in a gradient method.

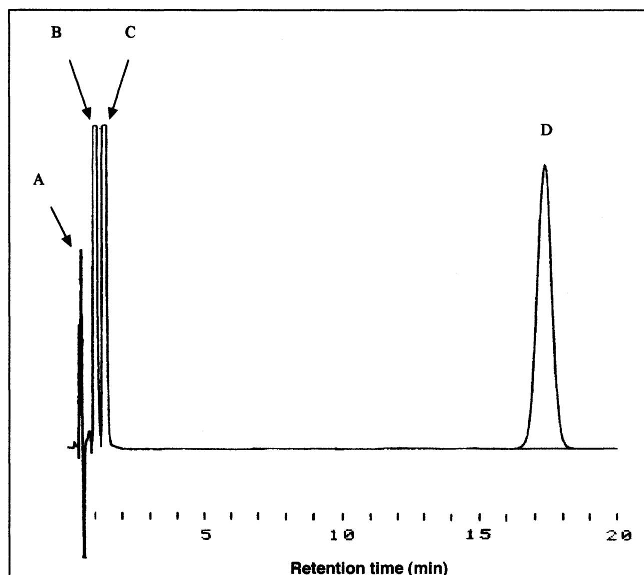

Figure 2. Chromatogram of the injection peak (A), calcipotriol (B), calcitriol (C), and cholecalciferol (D) on a LiChroCART RP18 column using a methanol–acetonitrile–water (67:23:10, v/v) mobile phase. Flow rate, 2 mL/min; wavelength, 254 nm; injection concentration, 100 µg/mL; loop volume, 20 µL.

Assay of Calcipotriol

With calcipotriol as the substance of interest and its elution close to the injection peak using a mobile phase containing methanol–acetonitrile–water (67:23:10, v/v), a new search was initiated to find a mobile phase that gave an elution further from the injection peak. Since quantitative analysis was to be conducted, UV detection was now monitored at 263 nm, based on a

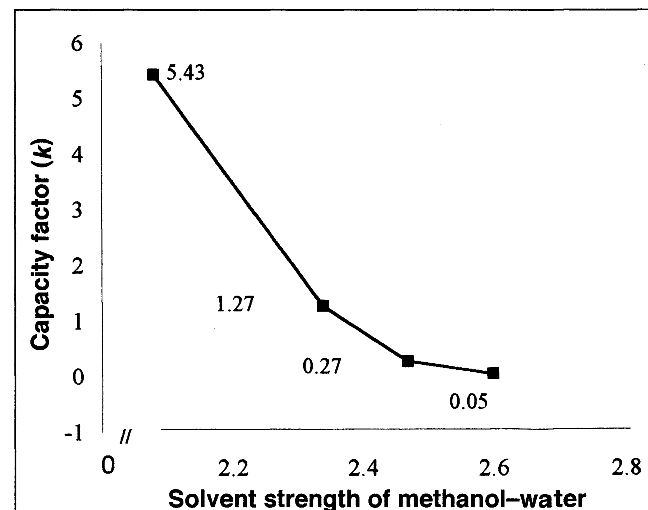

Figure 3. Summary of the effect of the organic modifier and the solvent strength on the capacity factor of calcipotriol. Column, LiChroCART RP18 (5 µm) 4 x 125-mm; mobile phase, methanol–water (80:20, v/v).

spectrophotometric analysis of calcipotriol. The mobile phase containing methanol–water (80:20, v/v, solvent strength 2.08) gave a k' value of 5.43, and thus no further search was conducted (Figure 3). Using this mobile phase, a parallel study was conducted to see if a better separation between the closely eluting analogues was also achieved. Results showed a good separation for calcipotriol and calcitriol with respective k' values of 5.43 and 10.32 and a resolution of 2.95. However, the mobile phase with the binary mixture of methanol–water (80:20, v/v) can only be used when cholecalciferol does not appear in a given sample; otherwise, it is advisable to use the ternary mixture of methanol–acetonitrile–water (67:23:10, v/v) described earlier.

Limit of detection and limit of quantitation

With a very low therapeutic dose for calcipotriol (5), and a relatively high risk of hypercalcemia if the recommended dose is not adhered to (16), another important aspect of the chromatographic process was studied: the sensitivity of detection. The relative sample sensitivity of a detector is defined as the minimum concentration of solute which can be detected and is evaluated by using the signal-to-noise (S/N) ratio. The S/N ratio can be calculated for the equation (17):

$$S/N = \frac{2H}{hn} \qquad \text{Eq 2}$$

where H is the height of the peak of interest (calcipotriol) and hn is the absolute value of the longest noise amplitude fluctuation from the baseline in a chromatogram obtained after injection of a blank solution, observed over a distance equal to 20 times the width at half height of a calcipotriol peak, and situated equally around the retention time of this calcipotriol peak.

Limit of detection

The limit of detection (LOD) was evaluated by six injections of a 10-ng/mL solution of reference calcipotriol at a preferred detection wavelength of 263 nm and a flow rate of 1 mL/min. The S/N ratio was 3.2. Results showed that calcipotriol can be easily detected, but the peak areas obtained were variable. The percent relative standard deviation was 3.71 (Table II).

Table II. Statistical Profile of the Peak Areas for the Identification of the Limits of Detection and Quantitation

Statistics	Calcipotriol 10 ng/mL	Calcipotriol 20 ng/mL
Injections	6	6
Peak area range	602–673	1132–1199
Mean	640.2	1180.7
Standard deviation	23.74	24.86
Relative standard deviation (%)	3.71	2.10
S/N	3.2	4.8

Limit of quantitation

The limit of quantitation (LOQ) was investigated by injecting 20 ng/mL calcipotriol. Six injections were performed and detected under the same conditions as LOD. With a S/N ratio of 4.8 and percent relative standard deviation of 2.10, the solution with 20 ng was considered the LOQ.

Linearity

The linear dynamic range, which is the number of times the sample concentration can be increased from the LOQ to the concentration at which the detector is no longer linearly proportional to the sample concentration, was studied. The relationship obtained between peak area and amount of calcipotriol injected is linear from the selected range of 20–100 ng/mL with a correlation coefficient of 0.9994. To verify the prediction capability, three concentrations of 25, 50, and 75 ng/mL situated in the linear dynamic range and within the standards to construct the calibration line were selected and injected. Results showed that the percent deviation of the peak areas obtained from the three prediction concentrations were ± 4% (Table III).

Table III. Predictability of Selected Points Located Within the Linearity Range of 20–100 ng/mL

Known concentration (μg/mL)	Measured area	Predicted area by calibration line	% Deviation
25	1565.67	1507.57	–3.71
50	2978.67	3044.68	2.21
75	4670.67	4581.79	–1.90

Determination of calcipotriol in a pharmaceutical preparation

The selection of solvent for the isolation of calcipotriol from its pharmaceutical preparation was based on finding the solvent which could dissolve most of the ingredients from the ointment (particularly calcipotriol). The principle behind the selection was to dissolve as much calcipotriol in the fatty fraction of the ointment as possible. Several solvents were tested by dissolving 1 g of ointment in 10 mL of solvent and shaking for 1 h. Solvents tested were methanol, ethanol, isopropanol, acetonitrile, and chloroform. The results showed that chloroform liquified the ointment while other solvents did not, even after prolonged shaking. Based on this observation, chloroform was selected to be the solvent of choice. The mixture produced after shaking was turbid and was therefore centrifuged at –5°C in order to separate the solidified fats and insolubles. The turbidity was caused by the presence of polyoxyethylene-2-stearyl ether, which is insoluble in chloroform and occurs in the ointment base. An intermediate step with methanol was necessary in order to avoid solubility problems between chloroform and the mobile phase. The mobile phase which was used as the final diluting solvent contained 80% methanol and 20% water. No separation in two mixtures (layers) was observed after the sample diluted with methanol was further diluted with the mobile phase. However, the solution became slightly turbid. This turbidity was caused by the insolubility of remaining fats in the aqueous solvent. No additional attempt was made to remove these fats, because calcipotriol is lypophilic and may be affected. The turbid solution was directly injected onto the column. No disturbances of the chromatograms by fats were observed. In order to minimize an occasional cumulative effect of these fats on the performance,

the column was rinsed with methanol for 30 min after turning the column to wash away the fats occasionally retained on the column. It was noted that the recovery was greater than 98%, so the cleaning regime administered to the column was efficient (or the fats did not cause any problems).

Recovery

The samples obtained from the determination of calcipotriol in the ointment were diluted for injection (using the same dilution scheme) to theoretical concentrations of 500 and 1000 ng/mL. The calcipotriol concentrations obtained from the ointment were compared to the peak areas obtained from reference solutions of calcipotriol (i.e., 500 ng/mL and 1 µg/mL). Tests for repeatability of injections and dilutions for both samples and standards at the two concentrations showed an overall relative standard deviation of less than 2%. When the peak areas obtained from the sample were superimposed on the peak areas of the reference, there were respective overlaps denoting high recovery. The recovery from six separate determinations in both concentrations were found to be greater than 98%. The complete procedure from isolation to recovery can be performed within 2 h.

Conclusion

The separation procedure for calcipotriol, calcitriol, and cholecalciferol can be used routinely as an additional control for the presence of related substances in the testing of raw material for any of the three Vitamin D_3 compounds. The simple assay procedure for calcipotriol can be used to detect its presence in samples, even at very low concentrations. The isolation and recovery can be performed within a couple of hours. These various methods described are simple, sensitive, repeatable, and can be used on a daily basis as part of routine analysis.

Acknowledgment

The authors wish to thank Schering AG (Berlin, Germany) and Leo Pharmaceutical Products (Ballerup, Denmark) for providing free samples of the Vitamin D_3 analogues. The authors would also like to thank Prof. Dr. D.L. Massart and staff of the Department of Pharmaceutical and Biomedical Analysis, where the HPLC work was conducted. This work was part of an oral presentation in the Zevende Forum der Farmaceutische Wetenschappen, April 24–25, 1997 in Blankenberge, Belgium.

References

1. J. Harris. *Vitamins in Theory and Practise,* 4th ed. University Press, Cambridge, 1955, pp 33, 157–197.
2. D. Fraser. Vitamin D. *The Lancet* **345:** 104–107 (1995).
3. D. Murdoch and S. Clissold. Calcipotriol. A review of its pharmaceutical properties and therapeutic use in psoriasis vulgaris. *Drugs* **43:** 415–29 (1992).
4. K. Kragbelle. Calcipotriol: a new drug for topical psoriasis treatment. *Pharmacol. Toxicol.* **77:** 241–46 (1995).
5. C. Debaille. Calcipotriol, nieuwe therapie voor psoriasis. *Het Apothekersblad* **2:** 29–32 (1994).
6. K. Kragbelle and L. Iversen. Calcipotriol. A new topical antipsoriatic. *Dermatol. Clin.* **11:** 137–41 (1993).
7. A. Kissmeyer and L. Binderup. Calcipotriol (MC 903): pharmacokinetics in rats and biological activities of metabolites. A comparative study with 1,25-$(OH)_2D_3$. *Biochem. Pharmacol.* **41:** 1601–1606 (1991).
8. H. Sorensen, L. Binderup, M. Calverly, L. Hoffmeyer, and N. Andersen. In vitro metabolism of Calcipotriol (MC 903), a Vitamin D analogue. *Biochem. Pharmacol.* **39:** 391–93 (1990).
9. M. Vargas-Ruiz, L. Bushman, M. Hillegas, and C. Halstenson. Stability of parenteral calcitriol in a tuberculin syringe. Abstract. *ASHP Midyear Clinical Meeting* **29:** P-243 (1994).
10. United States *Pharmacopoeia,* 22nd revision. US Pharmacopeial Convention, Rockville, MD, 1990, pp 303–304.
11. M. Amin. High performance liquid chromatography of fat-soluble vitamins. III. Determination of Vitamin D_3 in pharmaceutical preparations and in blood. *J. Chromatogr.* **11:** 1347–57 (1988).
12. L. Snyder. Classification of solvent properties of common liquids. *J. Chromatogr. Sci.* **16:** 223–34 (1978).
13. G. Doelman. Calcipotriol (Daivonex). *Pharm. Weekblad* **128:** 644–46 (1993).
14. J.L. Glajch, J.J. Kirkland, and K.M. Squire. Optimization of solvent strength and selectivity for reversed-phase liquid chromatography using an interactive mixture-design statistical technique. *J. Chromatogr.* **199:** 57–79 (1980).
15. R. Lehrer. The practice of high performance LC with four solvents. *International Laboratory* **November/December:** 76–88 (1981).
16. J. Bourke, J. Berth-Jones, R. Mumford, and P. Hutchinson. High dose topical calcipotriol consistently reduces serum parathyroid hormone levels. *Clin. Endocrinol. Oxf.* **41:** 295–97 (1994).
17. *European Pharmacopoiea,* 2nd ed., Part II. Council of Europe, Maisonneuve SA, Sainte-Ruffine, France, 1995, p V.6.20.4.

Manuscript accepted May 13, 1998.