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Technical Note

In Vitro Diffusion Cell Design and Validation. I. A Stability-Indicating High-Performance Liquid Chromatographic Assay for Betamethasone 17-Valerate in Purified Isopropyl Myristate Receptor Phase

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INTRODUCTION

The development of a reliable *in vitro* permeation system necessitates the use of a precise and accurate method of quantifying the amount of permeant partitioning from the membrane into the cell receptor phase. Aqueous donor and receptor chamber fluids have been used in the majority of reported investigations, which makes quantitative permeant analysis relatively facile. Alternatively, radiolabelled diffusants have been used and flux rates monitored by scintillation counting, obviating the need for chromatographic separation of the receptor-phase components. However, this technique is not applicable when nonlabelled compounds or commercial dosage forms are to be evaluated by a cell system. Furthermore, several studies indicate that aqueous receptor phases may not present an optimal partitioning environment for certain lipophilic permeants (1–4), thereby impairing accurate flux monitoring due to limited diffusant solubility. Several attempts have therefore been made to improve the partitioning environment within these systems, by the addition of surfactants for example (4). A lipophilic receptor environment appears beneficial for corticosteroid partitioning, and thus, the use of isopropyl myristate has been investigated because of its bipolar properties that tend to mimic the biochemical composition of the skin (5,6). Betamethasone 17-valerate and its 21-valerate degradation product are highly soluble in isopropyl myristate and this nonaqueous solvent will not augment C-17-to-C-21 ester degradation reactions.

The UV absorbance of the receptor-phase solutions has been measured directly by spectrophotometry without prior chromatographic separation (7–10). However, compounds other than the diffusant of interest may permeate from the donor compartment, or may leach from the membrane, and may contribute to this measured absorbance. Therefore, chromatographic separation of the receptor-phase constituents is considered essential for accurate analytical estimation and to distinguish degradation products or other UV-absorbing moieties. Although Poulsen *et al.* (5) have used thin-layer chromatography to separate corticosteroids in isopropyl myristate, high-performance liquid chromatography appears to be the analytical technique of choice for accuracy and precision. The developed methodology is similar to that described previously for the assay of betamethasone valerate esters in topical formulations (11) and uses a multidimensional, column-switching technique for isolation of the drug moieties and internal standard from the lipid phase, the latter presenting significant chromatographic problems without adequate separation.

MATERIALS AND METHODS

Reagents and Chemicals

The acetonitrile and methanol were glass distilled and spectral grade (Burdick and Jackson, U.S.A.). The high-performance liquid chromatographic-grade water used in the mobile phase was purified by a Milli-Q system (Millipore, Bedford, Mass.), and the mobile phase filtered through a 0.45-μm membrane filter (Type BD, Millipore). All chemicals were analytical reagent grade and were used as received. The betamethasone 17-valerate was obtained from Glaxo, South Africa; the norethisterone was from Ethnor, South Africa; and the betamethasone 21-valerate was an authentic specimen from the British Pharmacopoeia Commission. The isopropyl myristate (Merck, West Germany) and the propylene glycol (Saarchem, South Africa) were both synthesis grade, rated at 98 and 95% pure, respectively. Samples of higher purity were not available commercially.

Apparatus

The high-performance liquid chromatograph consisted of a solvent delivery system (Model 6000A, Waters, U.S.A.); a syringe-loading, six-port sample injector (Model

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7125, Rheodyne, U.S.A.) equipped with a loop column (MPLC, 3 cm × 4.6-mm i.d., packed with Lichrosorb RP-18, Brownlee, U.S.A.) and a variable-wavelength UV detector (Model SF769, Kratos Analytical Instruments, U.S.A.) coupled to a strip chart recorder (Model 100A, Perkin–Elmer, Palo Alto, Calif.) or an integrator (Model 3390A, Hewlett–Packard, Palo Alto, Calif.). A diode-array UV detector (Model 1040A, Hewlett–Packard) was employed in place of the variable-wavelength detector for spectral analysis. The analytical column (25 cm × 4.6-mm i.d.) was custom packed with octadecylsilane (Techsil 10 μm, HPLC Technology, U.K.). Valve switching and loop column flushing were accomplished manually.

Chromatography Conditions

The ion-free mobile phase was prepared by adding 45 parts by volume of water to 55 parts acetonitrile in a stoppered flask and allowing the mixture to equilibrate to room temperature prior to simultaneous filtering and degassing under vacuum. The mobile-phase flow rate was set at 1.5 ml/min and all chromatography was carried out at ambient temperature with the detector wavelength set at 239 nm.

Purification of the Isopropyl Myristate

To assess its purity, an aliquot of isopropyl myristate was extracted with an equal volume of mobile phase, and 10 μl of the latter injected into the chromatograph. Large absorption peaks between 4.5 and 6.5 min were presumably due to impurities in the lipid. An extraction of the isopropyl myristate with mobile phase containing betamethasone 17-valerate and norethisterone in solution (Fig. 1) indicated that, while it may be possible to resolve the norethisterone peak from the large impurity band, betamethasone 17-valerate eluted between the two impurity peaks, making adequate resolution impossible. Spectral analysis of the impurity peaks at 4.95 and 5.88 min (Fig. 2) indicates that both peaks have a UV absorbance maximum at approximately 225 nm, implying that these peaks are probably not due to impurities of steroidal origin. Similar spectral analysis of the betamethasone 17-valerate and norethisterone peaks demonstrate the indicative steroidal absorbance maximum at 240 nm.

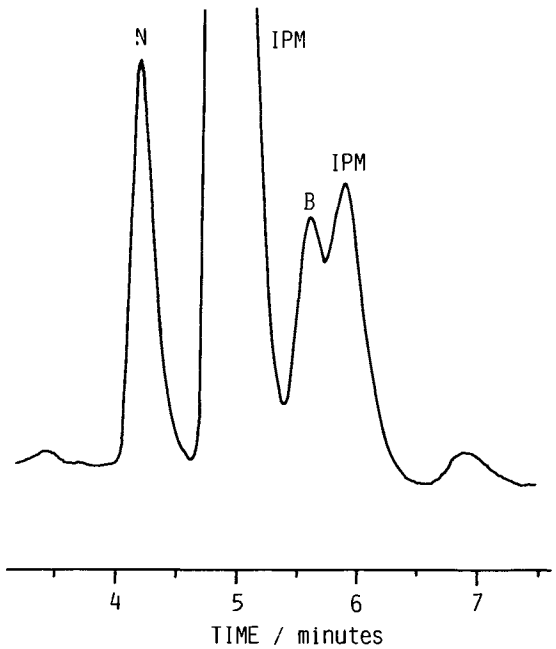

Fig. 1. Typical chromatogram of an unpurified isopropyl myristate extract showing elution interference of the impurities with the betamethasone 17-valerate peak. B, betamethasone 17-valerate; IPM, impurities in isopropyl myristate; N, norethisterone.

Purification of large volumes of isopropyl myristate by successive extractions with either acetonitrile or water was not feasible, as the former solubilized some of the lipid phase with each extraction, and the impurities did not partition into the aqueous phase. Successive extractions of the isopropyl myristate with propylene glycol aliquots significantly diminished the concentrations of impurities, and adequate removal for chromatographic sensitivity was effected by 15 isovolumetric extractions conducted in large volume separating flasks with centrifugation of the lipophilic layer. This method was adopted for the purification of all synthesis-grade isopropyl myristate used in subsequent *in vitro* permeation experiments.

Internal Standard Solutions

The internal standard solution was prepared in mobile phase at a norethisterone concentration of 3.5 μg/ml. Under the operating conditions described above it was found that the chromatographic system adequately resolved the norethisterone (4.2 min) from betamethasone 17-valerate (5.6 min) and betamethasone 21-valerate (6.5 min), together with the preservatives chlorocresol (3.6 min) and methyl hydroxy benzoate (2.9 min) contained in some commercial topical formulations evaluated by the *in vitro* diffusion cell. Resolution of the internal standard peak from enhanced solvent fronts due to donor solutions of corticosteroid in alcoholic solution was also achieved.

Calibration Standard Solutions

Standard stock solutions of betamethasone 17-valerate were prepared in purified isopropyl myristate at concentrations of 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, and 40.0 μg/ml. These solutions were used to test for the linearity of detector response and recovery characteristics of the corticosteroid from the isopropyl myristate. Similarly, in order to quantify any degradation product, solutions of betamethasone 21-valerate were prepared in isopropyl myristate at concentrations of 0.5, 1.0, 1.5, and 2.1 μg/ml. These ranges represented the expected magnitudes of the permeants in the receptor cell solution.

Sample Preparation

During the course of the permeation experiment, 40-μl aliquots were sampled from the calibration standard stock solutions or were withdrawn from the receptor chamber of the diffusion cell at specified time intervals using a fixed-volume transfer pipette (Brand, West Germany). These samples were placed into small glass collection tubes (4 mm-i.d. × 50-mm length), the tubes were covered (Parafilm, Amer-

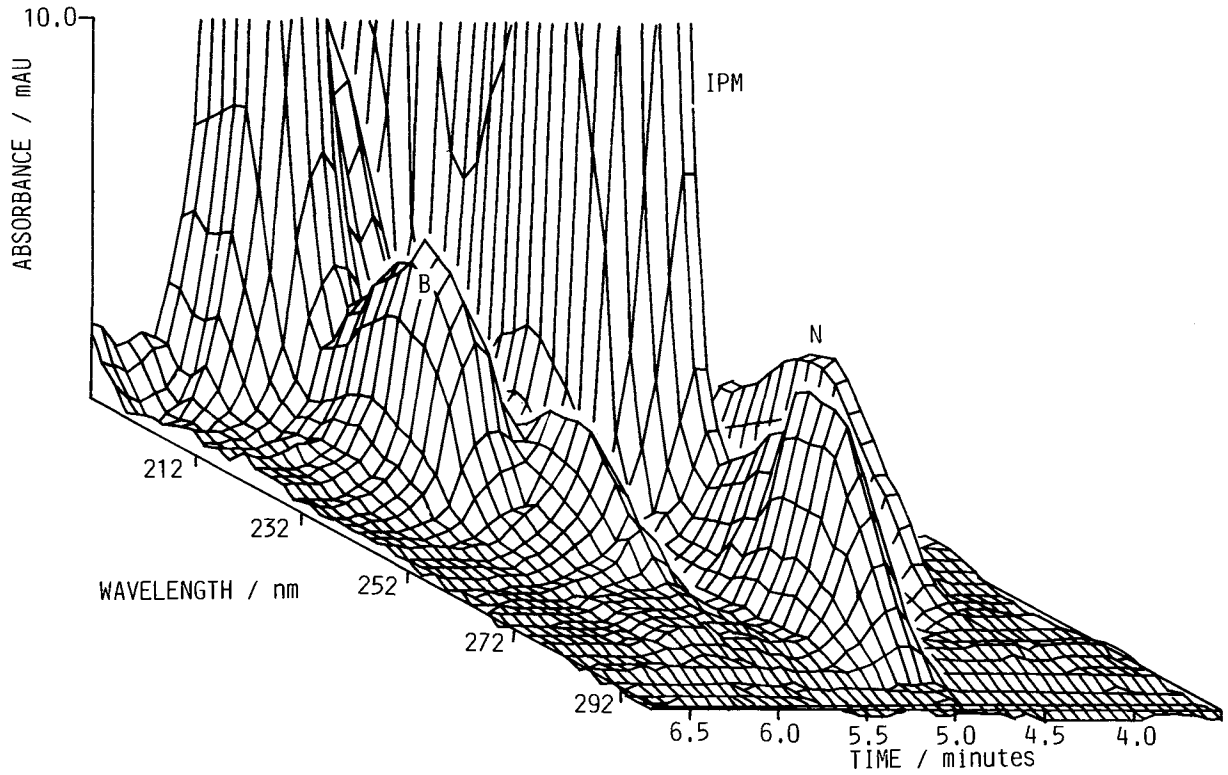

Fig. 2. Three-dimensional spectral plot of betamethasone 17-valerate (B), impurities in isopropyl myristate (IPM), and norethisterone (N).

ican Can Co., U.S.A.) and stored under refrigeration (4°C) until assayed. Prior to injection, 40 μl of internal standard solution was added to the isopropyl myristate aliquot in each tube and the mixture vortexed for 30 sec, ensuring a homogeneous, fine dispersion of the two immiscible phases and thereby facilitating optimal corticosteroid partitioning between the phases. The samples were then centrifuged for 60 sec to assist in the separation of the phases and 10 μl of the lower, mobile phase layer was withdrawn by syringe for injection into the chromatograph.

Injection Procedure

The sample injection and column-switching procedure was similar to that described previously (11). Lipophilic components trapped on the loop column were forward flushed to waste using 1 ml methanol followed by 5 ml mobile phase, thereby no reequilibration of the loop column was required prior to the next injection. It was found that methanol washes were adequate for displacing bound lipophilic components from the loop column, and the disruptive effects of using a stronger eluent such as tetrahydrofuran (12–14) were, therefore, avoided. Calibration standard solutions were interspaced between sample injections such that each solution was injected at least three times for means to be calculated. Peak height ratios from injected samples were compared to the ratios from calibration standard solutions to quantify the permeant concentrations using the relative response factor method (15,16).

RESULTS AND DISCUSSION

Figure 3 depicts chromatographic traces of the isopropyl myristate receptor solutions obtained from diffusion runs using different, commercial, betamethasone 17-valerate-containing topical formulations (Betnovate, Glaxo, South Africa) as the donor phase and human stratum corneum as the diffusion membrane. A typical chromatogram of the isopropyl myristate extract from a cream diffusion experiment is shown in Fig. 3a and exemplifies the resolution between the formulation preservative (chlorocresol) and the norethisterone internal standard. Figure 3b is a chromatogram from a lotion permeation experiment and demonstrates the large absorption band generated by the methyl hydroxy benzoate preservative. Figures 3c and d show chromatographic traces from ointment and scalp application permeation experiments, respectively; in both these cases there are no preservatives in the dosage forms. The presence of betamethasone 21-valerate fractions was rarely observed during routine chromatography. In instances when the degradation product was detected during assay of the topical dosage forms prior to initiating the permeation experiment, this moiety was also observed and quantified in the receptor chamber isopropyl myristate.

Specificity and Linearity Studies

To ensure the specificity of the detector response a UV spectrum was recorded at half-second intervals as the internal standard and betamethasone 17-valerate eluted from the

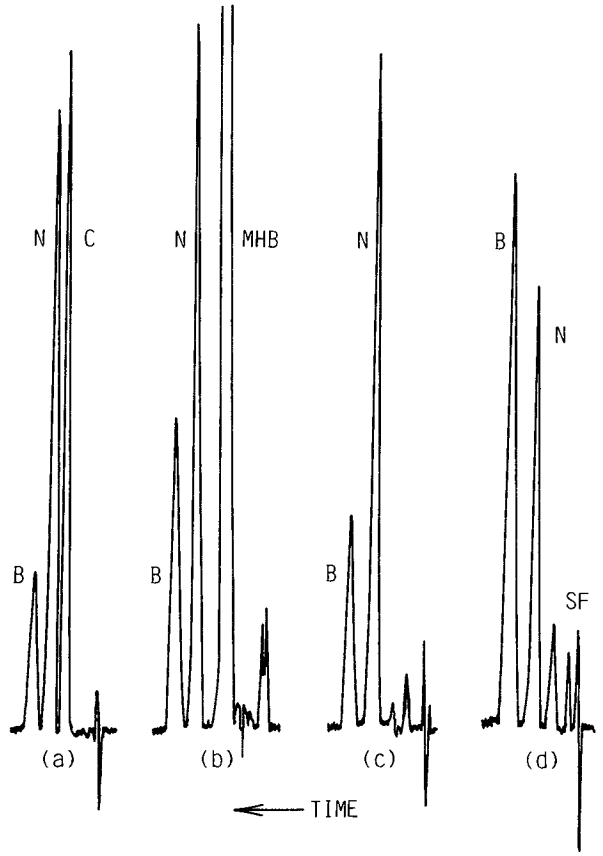

Fig. 3. Typical chromatographic traces from extracts of isopropyl myristate receptor phases from cream (a), lotion (b), ointment (c), and scalp application (d) permeation experiments. B, betamethasone 17-valerate; C, chlorocresol; MHB, methyl hydroxy benzoate; N, norethisterone; SF, alcoholic solvent front.

analytical column after sample aliquot injection. These spectra were compared to those obtained after injection of a pure solution of each compound. No obvious differences were noted, implying that the steroid peaks obtained from the extract of the lipid receptor solution were not augmented by any other extraneous constituents. Calibration curves (Table I) constructed on the basis of the peak height ratios of betamethasone 17-valerate/internal standard versus betamethasone 17-valerate mass were linear over the concentration range studied (2.5–40.0 μg/ml). Similar linearity was obtained for the betamethasone 21-valerate calibration curve over the concentration range 0.5–2.1 μg/ml, indicating that the assay method is suitable for quantitative purposes over wide corticosteroid concentration ranges and is sensitive to trace amounts of the permeants.

Table I. Linearity Data and Calibration Curve Construction[a]

Compound	Internal standard	Slope ×1000	Intercept ×100	Correlation coefficient	*N*
B 17-V	NOR	2.680	1.343	0.9992	30
B 21-V	NOR	1.371	0.295	0.9958	16

[a] B 17-V, betamethasone 17-valerate; B 21-V, betamethasone 21-valerate; NOR, norethisterone.

Precision, Accuracy, and Recovery Studies

To determine the precision, accuracy, and recovery of the method, solutions of betamethasone 17-valerate were made up in normal internal standard solution at concentrations of 5.0, 10.0, 20.0, 30.0, and 40.0 μg/ml. The average percentage recovery observed over this spike concentration range was 99.78% (SD, 1.68%; $N = 4$ samples at each concentration), indicating that negligible loss of betamethasone 17-valerate is experienced during sample preparation and that preferential partitioning of the corticosteroid occurs from the isopropyl myristate solution into the acetonitrile/water phase. The method is, therefore, sufficiently accurate and precise for analytical purposes.

On-line Cleanup

The column-switching technique was found to be highly suitable and efficient for the on-line cleanup of samples and effected adequate isolation of the lipophilic fraction from the analytical system, thereby obviating chromatographic interference and minimizing sample handling. Furthermore, deterioration of the analytical column performance was not experienced even after a year of continuous use.

CONCLUSIONS

The high-performance liquid chromatographic technique developed is highly efficient for the analysis of betamethasone 17-valerate concentrations in isopropyl myristate used as the receptor phase of *in vitro* diffusion cells. Extraction of the drug moiety from the lipid phase is effected by a combination of liquid–liquid extraction and on-line sample cleanup using a switching valve and loop column assembly. The method is shown to be relatively simple, accurate, and linear over the broad permeant concentration range experienced in diffusion experiments and has the capability of simultaneously quantifying degradation products and topical formulation preservatives in the cell receptor solution. The major advantage of having this reliable analytical technique available is that it allows the accurate monitoring of nonradiolabelled corticosteroid permeation in laboratory diffusion experiments, thereby allowing the testing of commercial products. Slight modifications to the methodology have allowed the assay of other corticosteroids, such as hydrocortisone, indicating that the technique is applicable to a diverse range of corticoid permeants.

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REFERENCES

1. T. J. Franz. *J. Invest. Derm.* 64:190–195 (1975).
2. R. C. Scott, D. E. Leahy, J. R. Evans, and E. Bole. *J. Pharm. Pharmacol.* 38: (Suppl.):66P (1986).
3. K. H. Valia and Y. W. Chien. *Drug Dev. Ind. Pharm.* 10:951–981 (1984).

4. R. L. Bronaugh and R. F. Stewart. *J. Pharm. Sci.* 73:1255–1258 (1984).
5. B. J. Poulsen, E. Young, V. Coquilla, and M. Katz. *J. Pharm. Sci.* 57:928–933 (1968).
6. F. Broberg, A. Brodin, B. Akerman, and S. G. Frank. *Acta Pharm. Suec.* 19:229–240 (1982).
7. L. Turakka, T. Piepponen, and P. Kahela. *Labo-Pharma-Probl. Tech.* 32:540–544 (1984).
8. K. B. Sloan, K. G. Siver, and S. A. M. Koch. *J. Pharm. Sci.* 75:744–749 (1986).
9. L. Turakka, K. Kontra, and A.-M. Kyllonen. *Pharm. Acta Helv.* 58:153–159 (1983).
10. T. J. Roseman. *J. Pharm. Sci.* 61:46–50 (1972).
11. E. W. Smith, J. M. Haigh, and I. Kanfer. *Int. J. Pharm.* 27:185–192 (1985).
12. D. L. Conley and E. J. Benjamin. *J. Chromatogr.* 257:337–344 (1983).
13. J. A. Apffel, T. V. Alfredson, and R. E. Majors. *J. Chromatogr.* 206:43–57 (1981).
14. R. A. Kenley, S. Chaudhry, and G. C. Visor. *Drug Dev. Ind. Pharm.* 11:1781–1796 (1985).
15. K. Hammarstrand. *Varian Instrum. Appl.* 10:10–11 (1976).
16. L. M. Upton, E. R. Townley, and F. D. Sancilio. *J. Pharm. Sci.* 67:913–916 (1978).