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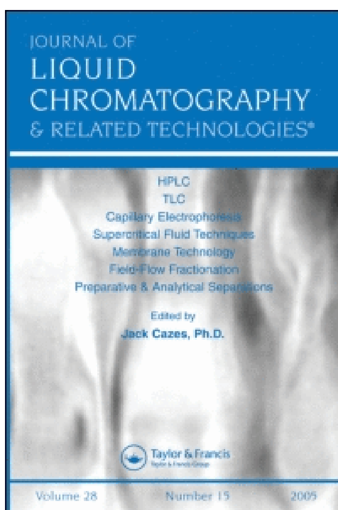

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THE VALIDATION OF A NEW STABILITY-INDICATING HPLC TECHNIQUE DEVELOPED FOR THE QUANTITATIVE ANALYSIS OF ROOPEROL TETRA-ACETATE

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ABSTRACT

A high performance liquid chromatographic method was developed for the quantitative analysis of rooperol tetra-acetate in the aqueous receptor phase of *in vitro* diffusion cells. System validation of the developed HPLC technique was carried out through the determination of the precision, limit of quantitation, sensitivity, linearity, and range of the system. In each of the above tests mentioned, an isocratic mobile phase of acetonitrile/water facilitated the elution of rooperol tetra-acetate dissolved in acetonitrile. Experimental variations were controlled through the use of the internal standard, prazepam. The precision results, expressed as the percent relative standard deviation, ranged from between 1.35% to 4.23%.

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The results obtained in all other tests performed were acceptable thus verifying that the analytical method used is of good design. Further investigations on rooperol tetra-acetate involved performing stability tests, thus chromatographically characterizing the degradation process of the drug. Heat degradation of rooperol tetra-acetate, when stored in a glass vial and a plastic holder, was assessed over a twelve week period. Data evaluation involved the application of the t-distribution test, used to determine if a significant difference, at a 95% confidence level, exists between the drugs stored under heat and a sample stored at room temperature.

INTRODUCTION

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Various clinical studies on the aglycone of hypoxoside, namely rooperol, have successfully demonstrated the application of the agent as a cytotoxic substance.[1,2,3] The hypoxoside, which has shown potential in the treatment of solar keratoses, is first extracted from the corms of *Hypoxis rooperi* and *H. latifolia*[4] before it is deconjugtated to rooperol using beta glucosidase. Rooperol, which is susceptible to oxidation, was stabilised by acetylating the compound to give rooperol tetra-acetate. The mechanism by which the drug effects its cytotoxic activity is believed to be through the disruption of chromosomal structures during the mitotic stage of cell development.[2] Problems associated with the delivery of clinical amounts of rooperol tetra-acetate into skin from a topical vehicle, necessitated the development of a quantitative assessment technique for the drug in diffusion assessment procedures. In order to achieve the release of rooperol tetra-acetate into the skin from a topical base, an effective drug delivery system needs to be developed. Before such a delivery system can be designed, a suitable quantitative assay method for the analysis of rooperol tetra-acetate is essential. Validation of the developed HPLC method involved performing precision, limit of quantitation, and range assays of the active drug combined with the internal standard and the assessment of replicate injections of this standard solution into the HPLC system. This method is useful in confirming the reliability and reproducibility of analytical applications selected for the study of rooperol tetra-acetate. Limited HPLC work has been previously performed on rooperol tetra-acetate and these studies concentrated on chromatographic analysis of the biotransformation of hypoxoside and rooperol analogues in man through the use of an in-line sorption enrichment HPLC technique. As system validation is an integral part of liquid chromatographic methods it was necessary to, firstly, design a suitable validated analytical method for the HPLC separation of rooperol tetra-acetate and, secondly, to establish the stability

profile for the drug in the analytical system.[5] Investigations on the stability of rooperol tetra-acetate involved subjecting the drug to elevated temperatures. The United States Pharmacopoeia guidelines for stability testing were used to determine the conditions under which accelerated tests were to be conducted.[6] The aim of this study is to describe the analytical technique used for validating the quantitative analysis of rooperol tetra-acetate and to generate a stability profile for the drug.[7]

MATERIALS

Reagents and Chemicals

A mobile phase of spectral grade acetonitrile obtained from Burdick and Jackson, USA and HPLC grade water purified through a Milli-Q system (Millipore, USA) was filtered through a 0.45μm membrane filter (type BD, Millipore, USA) and simultaneously degassed under vacuum at ambient temperature. Rooperol tetra-acetate was obtained from the Department of Pharmacology at Stellenbosch University, South Africa, and the prazepam was obtained from Parke-Davis (South Africa).

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Apparatus

A solvent pump, (Model SP8810, Spectra-Physics, California, USA) was connected to a manual injection valve (Model 7126, Rheodyne, California, USA) equipped with a 20μL injection loop. A 10μm C_{18} analytical column which was custom packed with octadecylsilane was used. The variable U.V. detector (Linear 200 model, Spectra-Physics, California, USA) which was set at a wavelength of 260nm and sensitivity of 0.002 A.U.F.S. was connected to a datajet integrator (Model SP4600, Spectra-Physics, California, USA). A hot box oven (Model 4B-5599-E, Gallenkamp, England) was used to carry out the accelerated stability tests at elevated temperatures.

HPLC Conditions

The mobile phase was prepared by mixing 70 parts acetonitrile with 30 parts water in a stoppered flask. The mixture was equilibrated to room temperature before being filtered and degassed. The flow rate was maintained at 1.5mL/min and all operations were carried out at ambient laboratory temperature.

METHODS

Precision Studies

At the beginning of each of the three precision runs performed, three individual samples of 0.01g rooperol tetra-acetate were weighed out and each sample was dissolved in acetonitrile in a 10mL volumetric flask. Each rooperol tetra-acetate sample was then diluted to a concentration of 0.1μg/mL in acetonitrile. The internal standard solution, which was diluted to a concentration of 1μg/mL in acetonitrile, was prepared by dissolving 0.025g prazepam in acetonitrile in a 25mL volumetric flask. A 2mL sample of the internal standard was added to a 2mL sample of rooperol tetra-acetate and 10μL of this final standard solution was injected into the HPLC system. A total of ten replicate injections, of each of the three standard solution samples prepared, were made on each of three occasions and the data obtained was used to calculate the percent relative standard deviation.

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Limit of Quantitation Studies

A 1000μg/mL solution of rooperol tetra-acetate dissolved in acetonitrile was prepared. Using the 1000μg/mL stock solution, six dilutions ranging in concentration from 0.501μg/mL to 1.002μg/mL were made. A 2mL aliquot of each dilution solution was mixed with 2mL of internal standard solution. A total of six 10μL replicate injections of the standard solution were introduced into the HPLC system. From the data obtained, the ratio of areas was determined and was used to calculate the mean and percent relative standard deviation. Data from the limit of quantitation studies was also used to plot a calibration curve for rooperol tetra-acetate and to determine the sensitivity and linearity.

Range Studies

Range studies were performed by preparing six samples of rooperol tetra-acetate in acetonitrile, the concentration of which covered the extremes of the range concerned. The masses of rooperol tetra-acetate weighed ranged from 14mg to 30.6mg. Suitable dilutions of each solution were made and a 2mL aliquot of the diluted solution was mixed with 2mL internal standard. Up to six replicate injections measuring 10μL each of standard solution were introduced into the HPLC system. The ratio of the areas was calculated from the data

obtained. The corresponding drug concentrations were calculated by solving the regression equation for concentration and substituting the peak areas ratio for the sample.

Stability Studies

A 1g sample of rooperol tetra-acetate was measured into a teflon plastic holder and the same amount was measured into a glass vial. Both containers were placed in the oven set at 40°C. At weekly intervals, a 0.1μg/mL solution of rooperol tetra-acetate in acetonitrile was prepared from each of the two samples stored in the oven. A 2mL measure of the rooperol tetra-acetate solutions was mixed with 2mL internal standard and six replicate injections of the mixture, each measuring 10μL, were introduced into the HPLC system. For the purpose of comparison, a 0.1μg/mL solution of rooperol tetra-acetate in acetonitrile was freshly prepared from a sample maintained at room temperature. Experimental manipulation of the fresh sample was identical to that of the samples stored at higher temperatures.

The retention times and areas of each peak produced were recorded. Statistical evaluation of the results collected over twelve weeks made it possible to assess the storage stability of rooperol tetra-acetate.

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RESULTS AND DISCUSSION

Precision Studies

Precision studies are concerned with the repeatability and reproducibility of a given method.[8] Most reported chromatographic methods have percent relative standard deviations of between 5% and 10% . However, the commonly accepted limit for inter-day RSD values for a given HPLC assay is less than 5%.

The percent relative standard deviation for the nine lots of injections had an overall average of 2.4% for all ninety injections. The percent relative standard deviation values for day one were 2.71%, 4.23%, and 1.42%; day two, 2.25%, 3.36%, and 1.45% and day three, 1.89%, 2.10%, and 2.33%. It is evident from the results that the assay method is sufficiently precise for quantitative analysis.

Limit of Quantitation Studies

Limit of quantitation studies are used to quantify the lowest concentration of drug that may be quantitatively assessed in a given sample.[9] Results for this test were determined by calculating the percent relative standard deviation of the instrument response for progressively lower concentrations of rooperol tetra-acetate. The lowest concentration quantified from the ratio of the areas was 0.0501μg/mL. However, at these low concentrations the percent RSD value was as high as 15.2%, thus indicating that the reliability of the system at this level is significantly reduced. The limit of quantitation was calculated to be 50.1ng.

Sensitivity Studies

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Sensitivity studies are dependent upon the degree of response, by the system, to concentrations of analyte covering the entire range of analysis.[9] The slope of the analytical calibration curve provides a measure of the sensitivity of the system. In the analysis of rooperol tetra-acetate, the slope and therefore, the sensitivity of the system was calculated as being 0.5727 (Figure 1). In addition, the width of the vertical bars of the calibration curve are an indication of the accuracy with which the system is able to respond to decreasing concentrations of analyte. The presence of narrow vertical bars indicates that the sensitivity of the system is satisfactory.

Linearity Studies

When examining the calibration curve, it is evident that the ratio of areas is proportional to the concentration of rooperol tetra-acetate present in the samples analysed. The correlation coefficient of the slope of the regression line which gives a measure of linearity was calculated to be 0.99765 (Figure 1). A variance value of one indicates a linear slope and, therefore the value obtained experimentally, certifies that the system is acceptably linear.

Range Studies

The results obtained during this test clearly indicate that both the system and method employed are reliable. Acceptable precision was obtained when the method used was applied to samples containing concentrations of analyte which encompass the entire range of analysis.[5] The percent deviations of the masses weighed range from -0.09% to +2.6% (Table 1).

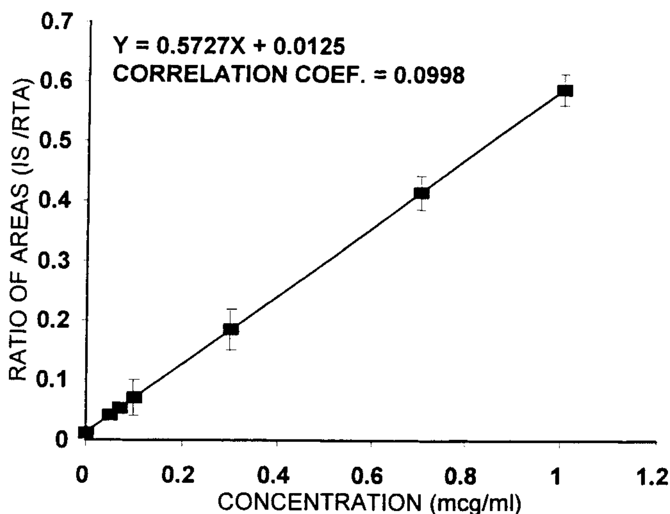

Figure 1. Calibration Curve for Rooperol Tetra-Acetate.

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Table 1

Range Data

Mass RTA Weighed (g)	Mass RTA Calculated (g)	Percent Standard Deviation
0.00014	0.000143	-2.1
0.00177	0.001798	-1.6
0.002276	0.002246	-0.037
0.00042	0.000409	+2.6
0.02209	0.021587	+2.3
0.03057	0.030596	-0.09

Stability Studies

The overall stability test results indicate that there was significant rooperol tetra-acetate degradation of both drug samples prepared following exposure to high temperatures during the three month period of analysis.

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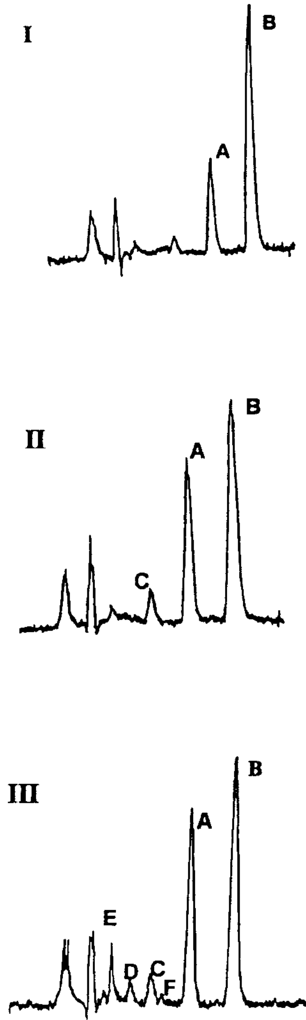

Figure 2. Typical Chromatograms of Rooperol Tetra-Acetate. (A) represents rooperol tetra-acetate; (B) represents the internal standard, prazepam; (C), (D) (E) and (F) are all degradation peaks. Chromatogram (I) was taken after two weeks of analysis, (II) and (III) after four and ten weeks respectivly.

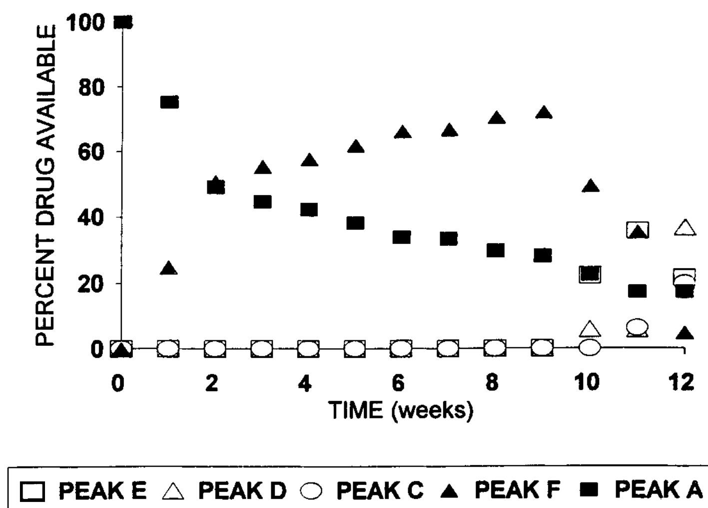

Figure 3. Stability profile illustrating the degradation of rooperol tetra-acetate in a glass vial. The graph shows the changes brought about by degradation of the sample during heat exposure. Initially, a single peak A was detected. With the progression of time, the appearance of additional peaks (C to F) were noted.

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Peaks C, D, E, and F (Figure 2) in both the plastic- and glass-stored samples were not present in the chromatograms of the freshly prepared samples, but began to appear in the degrading sample following three weeks of exposure of the plastic holder to elevated temperatures and ten weeks of exposure of the glass vial. Initially, a single peak (A) was detected (Figure 2[I]). However, with the progression of time, the appearance of additional peaks (C, D, E, F) signified the onset of the degradation process (Figure 2 [II and III]).

The stability profile of rooperol tetra-acetate displays a significant percentage mass decrease in peak A and a percentage mass increase in peaks C, D, E, and F in both the glass vial (Figure 3) and the plastic holder (Figure 4). Towards week twelve the baseline of the chromatogram was very rugged, thus, suggesting the emergence of several additional degradation products whose concentrations were far too low for the detector to quantitate. In addition, slight variations from week to week in the mobile phase composition, injection volume, pH, and temperature during chromatographic analysis could well have contributed to any decrease in the precision of the method. The more rapid onset of the degradation process observed with the plastic-stored sample may

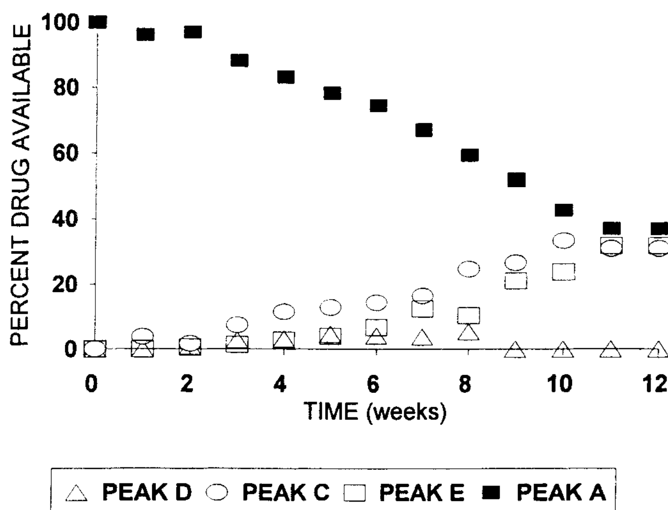

Figure 4. Stability profile illustrating the degradation of rooperol tetra-acetate in a plastic holder.

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possibly be due to unfavourable interaction of the drug with plasticizers released from the holder upon heat exposure. The glass vial used in the comparative study was inert and as a result, the rate of heat degradation of the drug was slower.

The student's *t* distribution test was applied to the peak area ratios obtained for all peaks and the results showed that at a 95% level, there is a significant difference in composition between the freshly prepared samples and the heated samples, thus confirming the instability of rooperol tetra-acetate in heat.

From the study of the structure of rooperol tetra-acetate (Figure 5), it is safe to presume that the metabolites formed when the drug is stored under elevated temperatures arise from successive cleavage of the acetate chemical groups. These acetate groups, situated on the periphery of the structure, experience decreased electronic attraction from the strong pi and triple bonds present at the core of the molecule, resulting in relatively easier detachment of the acetate groups from the core structure. Subsequent degradation may arise as a result of cleavage of the bonds which bind the two aromatic compounds of the rooperol tetra-actate structure.

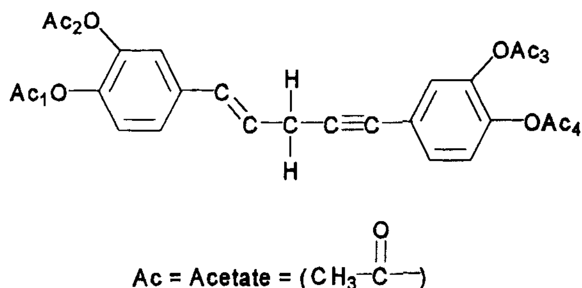

Figure 5. The Chemical Structure of Rooperol Tetra-Acetate (MW = 450.45g/mol).

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CONCLUSION

The precision, linearity, sensitivity, and range studies data indicate that the method selected is suitable for quantitative analysis of rooperol tetra-acetate. Furthermore, the technique has proven to be both reliable and reproducible. The method developed and validated can now be used to determine the diffusion characteristics of rooperol tetra-acetate through membrane systems.

The stability profile of rooperol tetra-acetate shows that the drug is significantly unstable and its degradation upon exposure to elevated temperatures for extended periods of time, is a significant property of the drug. The inherent instability of rooperol tetra-acetate at elevated temperatures, impacts on the manufacturing and diffusion processes where heat application may be necessary. For this reason, it is essential that the possible emergence of degradation peaks is acknowledged, particularly during the analysis of the diffusion properties of the drug.

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