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## Note

# High-performance liquid chromatographic method for the determination of sudexanox as the free acid in plasma

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Sudexanox, [5-(*n*-hexyl)-7-*S*-methylsulfonimidoyl)xanthene-9-one]-2-(tris-hydroxymethyl) carboxylate (RU-31156, I, Fig. 1), is a new xanthene derivative developed by Roussel UCLAF (Paris, France). This compound has a profile of anti-asthmatic properties similar to disodium chromoglycate but was found to be, in animal studies, about 100 times more potent and with the advantage that it can be given as an oral dose.

This paper describes a high-performance liquid chromatographic (HPLC) method based on a method originally developed by the Centre de Recherche, Roussel UCLAF, for the determination of sudexanox as the free acid [1] (RU-31122, II, Fig. 1) from plasma using sulthiame (III, Fig. 1) as internal standard and depicts the performance of this method during the analysis of samples generated by a clinical trial to evaluate the absolute and relative bio-availability of this drug from different formulations.

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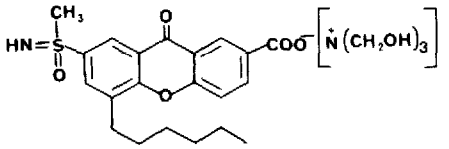
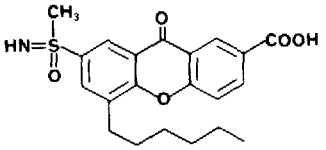


I



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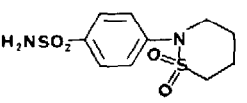

III

Fig. 1. Structural formulae for sudexanox, [5-(*n*-hexyl)-7-*S*-methylsufonimidoyl)xanthene-9-one]-2-(trishydroxymethyl) carboxylate (I), sudexanox free acid (II) and sulthiame (III).

## EXPERIMENTAL

### *Reagents*

Tetrabutylammonium hydrogen sulfate was supplied by Regis while all other reagents and solvents were of guaranteed reagent grade and supplied by E. Merck (South Africa). Water was double-distilled and filtered before use. Sudexanox was a gift from Roussel UCLAF and sulthiame was obtained from Bayer Miles (South Africa).

### *Mobile phase*

The mobile phase was isooctane—isopropanol—glacial acetic acid (68:28:4).

### *Buffer solution and ion-pair reagent*

The buffer solution was prepared by mixing 1.0 *M* solutions of $K_2HPO_4$ and $KH_2PO_4$ in the right proportions to obtain a solution of pH 7. To an aliquot of this solution, sufficient for the number of samples to be assayed on a particular day, was added a quantity of tetrabutylammonium hydrogen sulphate to result in a concentration of 1.5 mg of the ion-pair reagent in 200 $\mu$l of buffer.

### *Apparatus*

A Waters Model M600 solvent delivery system was used to deliver the mobile phase at a flow-rate of 2.5 ml/min to a $\mu$Bondapak ($NH_2$)® Radial-Pak® cartridge (10 $\mu$m, 10 cm × 8 mm) at room temperature. Injections were made via a Valco loop injector fitted with a 250-$\mu$l loop. Detection was done by means of a Waters Lambda Max Model 481 variable-wavelength UV detector operated at 250 nm and a sensitivity of 0.002 a.u.f.s. (all obtained from Millipore, South Africa). Chromatograms were recorded on a Radiometer Model REC 52 recorder (Radiometer, Copenhagen, Denmark).

### *Internal standard*

An amount of sulthiame was weighed out accurately, dissolved in the minimum amount of 0.1 *M* sodium hydroxide and diluted with water to obtain

a solution containing 200 ng of sulthiame per 50 μl. This amount was then used as internal standard for concentrations of II between 20 and 150 ng/ml, while a volume of 100 μl of the same solution was used for concentrations between 100 and 300 ng/ml. When stored at −20°C this solution showed no sign of deterioration during a period of three months.

*Sudexanox stock solutions*

Sudexanox was weighed out accurately and dissolved in methanol to obtain an initial solution containing 150 ng of the free acid (II) per 10 μl of methanol. This solution was then further diluted to obtain final solutions containing 15, 30, 45 and 75 ng of II per 10 μl. Aliquots of these solutions were flame-sealed in amber glass ampoules and stored at −20°C until needed. Plasma standard solutions equivalent to 30, 60, 90, 150 and 300 ng/ml II were then prepared by adding 10 μl of the relevant solution to 0.5 ml of drug-free plasma. This procedure had to be followed since it was found that the addition of larger volumes of methanol resulted in the appearance of undesirable peaks in the chromatogram. Methanol had to be used as solvent since sudexanox does not dissolve readily in water. The methanol solutions of sudexanox, when stored at −20°C, proved to be stable during the three-month test period.

*Extraction procedure*

To 0.5 ml pasma (calibration standards, controls or unknowns) were added the appropriate amount of internal standard solution (50 or 100 μl) and 200 μl of the 1.0 *M* phosphate buffer solution containing 1.5 mg of tetrabutylammonium hydrogen sulphate. The samples were shaken briefly to ensure thorough mixing and incubated at 4°C in a refrigerator for a period of 30 min. Dichloromethane (5 ml) was added and the samples were extracted for 15 min on a rotating shaker revolving at approximately 30 rpm.

After centrifuging in a cooled centrifuge at 4°C for 5 min at 2000 rpm (1000 *g*) the aqueous phase was removed by aspiration and the organic phase transferred to a disposable 5-ml glass ampoule. The solvent was evaporated to dryness under a stream of high-purity nitrogen and the residue dissolved in 200 μl of the mobile phase and the whole volume injected onto the column.

## RESULTS AND DISCUSSION

*Recovery of II from plasma*

The recovery of II from plasma was determined by adding 200 ng of this substance to 0.5 ml drug-free plasma and extracting it as described. Comparison of the peak height obtained with the peak height obtained when 200 ng was injected without an extraction procedure demonstrated a recovery of 90%.

*Quantitation*

A four-point calibration graph spanning the expected concentration range (30—150 ng/ml or 90—300 ng/ml) was constructed for each day's analysis. Quantitation was done by means of the internal standard method utilizing peak heights.

TABLE I

ACCURACY AND PRECISION OF METHOD FOR II

| Control No. | Actual concentration (ng/ml) | Mean concentration determined (ng/ml) | $n$ | Coefficient of variation (%) |
|---|---|---|---|---|
| Q1 | 183.4 | 189.7 | 4 | 0.8 |
| Q2 | 129.0 | 132.4 | 22 | 3.9 |
| Q3 | 90.2 | 90.6 | 9 | 6.8 |
| Q4 | 32.2 | 34.1 | 13 | 9.4 |

*Performance of the method*

The accuracy, precision and reliability of the method, after an initial validation, were evaluated in the following ways during the analysis of samples generated during a clinical trial. (a) Accuracy and precision were determined by the daily analysis of in vitro prepared control samples spanning the expected concentration range. At least two control samples of different concentrations were analysed each day. The results are presented in Table I. (b) The long-term reliability of the method was evaluated by the analyses of ex vivo quality-control samples generated as follows. During the clinical trial the plasma samples obtained from trial subjects were divided into two equal aliquots. One aliquot was used for analyses purposes while the other sample was stored as a back-up sample should the necessity of repeating some analysis occur. Some of these duplicate samples were recoded by the quality-control officer and two randomly selected samples were included in each day's analysis. The results of the first and second analyses of each of these samples were statistically analysed according to the Jackknife technique [2]. This technique, which takes into account that both variables are random, claims that two analyses are equivalent if (a) the fit is linear, (b) the deviation about the fit is small and (c) the slope of the regression line is close to unity. The confidence interval

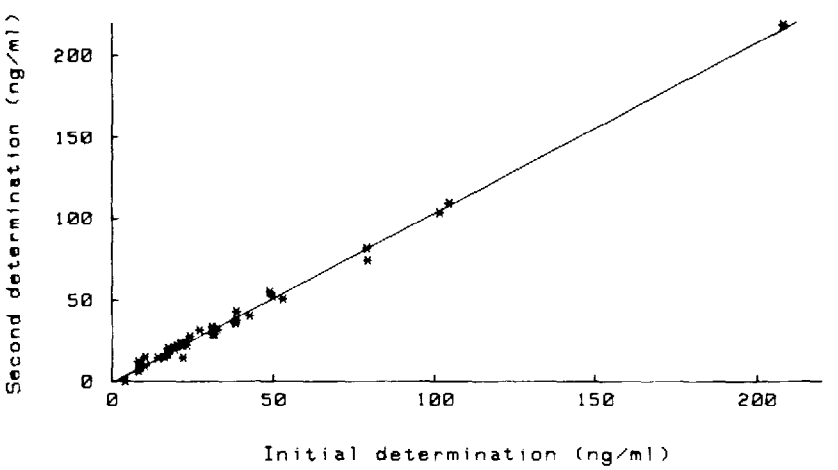


Fig. 2. Ex vivo quality control of the method during analysis of samples obtained during a clinical trial. $y = -1.293 + 1.046x$; $r^2 = 0.9939$.

($\alpha$ = 0.05) constructed for the slope of the linear regression line according to this technique (0.966; 1.142) reveals that the slope does not differ statistically from unity. The result of the statistical analyses of the results of the ex vivo quality-control samples are represented in Fig. 2. The regression line through these points shows a linear fit with an excellent correlation ($r^2$ = 0.994) and a slope of 1.047. Using conventional statistical methods, the confidence intervals ($\alpha$ = 0.05) for the slope and $y$-intercept of the linear regression line which are (0.953; 1.141) and (−6.544; 3.69), respectively, indicates that no statistical evidence could be found that the slope and $y$-intercept differ from unity and zero respectively.

The detection limit of the method (signal-to-noise ratio 2:1) was found to be 3.0 ng/ml).

The absence of any interfering peaks is illustrated in Fig. 3A while Fig. 3B and C shows the position of II in plasma without the addition of the internal standard and the relative positions of the analyte and the internal standard, respectively.

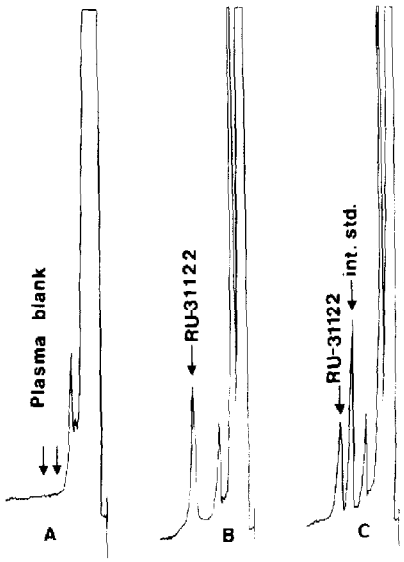


Fig. 3. Chromatograms of extracts showing (A) plasma blank, (B) plasma containing 60 ng/ml sudexanox (RU-31122) and (C) plasma containing sudexanox (40 ng/ml) and the internal standard, sulthiame.

*Column life*

Owing to the use of a highly acidic mobile phase it was found that the column deteriorated faster than would normally by expected. This deterioration manifests itself by the appearance of peaks that may interfere with the analyte peaks. This aspect of the assay procedure was monitored by the daily inclusion of a blank sample. Columns lasted for approximately 350 to 400 injections and were replaced as soon as these interfering peaks appeared.

*Alternative internal standard*

It was found that owing to variation in column characteristics during aging

some columns would loose their resolution between II and sulthiame. In such instances *p*-nitrobenzoic acid may be used as internal standard.

*Ion-pair reagent*

Extraction without the ion-pair reagent resulted in an extraction efficiency of about 50% (compared to 90% with the aid of the reagent) with a resultant loss in sensitivity. The use of this reagent also results in a cleaner extract than is the case when it is omitted.

*Volume of plasma used*

It was found that 0.5 ml plasma was the optimum volume for the procedure as described. The use of a larger volume of plasma (e.g. 1.0 ml) would result in a greater amount of II available for injection, but is was found that this volume of plasma resulted in a more than proportional increase in the height of peaks due to plasma impurities.

*Stability of sudexanox in plasma*

Samples stored at −20°C showed no signs of decomposition during a three-month period.

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2 J.N. Arvesen and M.W. Layard, in C.R. Buncher and J.-Y. Tsay (Editors), Statistics in the Pharmaceutical Industry, Marcel Dekker, New York, 1981.