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Investigation of the chemical equivalence of the trypanocidal products, Samorin® and Veridium®

J.N.A. Tettey [a], G.G. Skellern [a], M.H. Grant [b], J.M. Midgley [a,*]

[a] *Department of Pharmaceutical Sciences, SIBS, University of Strathclyde, 27 Taylor Street, Glasgow G4 0NW, UK*
[b] *Bioengineering Unit, Wolfson Centre, University of Strathclyde, 106 Rottenrow East, Glasgow G4 0NW, UK*

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Abstract

A procedure for the evaluation of chemical equivalence of proprietary formulations of isometamidium is described. The method combines the analysis of the principal component (isometamidium), HPLC profiling of related substances and determination of the inorganic impurity, ammonium chloride, using a modification of the Berthelot (Indophenol) reaction. Application of these procedures to analyses of commercially available sachets from four different batches of Samorin® and four different batches of Veridium® has demonstrated that there are marked qualitative and quantitative differences between batches from these two sources. Whilst Samorin® samples showed inter-batch consistency of composition, there was considerable inter-batch variation between the samples of Veridium®.

Keywords: Isometamidium; Samorin®; Veridium®; Ammonium chloride determination; Chemical equivalence; High performance liquid chromatography

1. Introduction

Since its introduction in 1958 [1], Samorin®/Trypamidium® (Rhône Merieux, Lyon, France) has remained the only agent available for the chemoprophylaxis of trypanosomiasis in animals, and has been used in affected areas worldwide [2]. Samorin® is defined as a mixture of isomers [3,4] with isometamidium (8-(3-*m*-amidinophenyl-2-triazeno)-3-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride, ISM, **II**) as the major component together with **I**, **III**, **IV** and **V** (Fig. 1). The pharmacological action has been attributed primarily to ISM [5,6]. The other isomers of which 7-(*m*-amidinophenyldiazo)-3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (M&B4250, **V**) is the most abundant also possess trypanocidal properties [6].

Samorin® is synthesised by the controlled coupling of diazotised *m*-aminobenzamidine monohydrochloride with 3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride (ethidium chloride, **I** (Fig. 1)) [1,7]. The coupling of a diazonium salt with an aromatic primary amine to give a diazoamino compound is an electrophilic substitu-

* Corresponding author.

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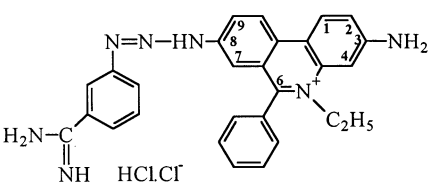
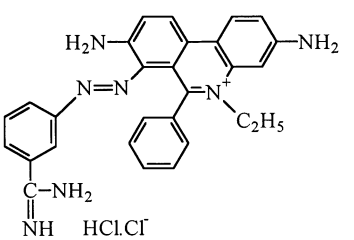
tion reaction involving the diazonium ion and the non-ionised amine. The presence of the amidino substituent enhances the reactivity of the parent benzenediazonium ion leading to an increase in the range of the coupling reaction with the resultant production of other isomers, notably **III**, **IV** and **V** [7]. Therefore the nature and extent of the coupling is influenced by careful control of the reaction conditions, notably the temperature and pH of the reaction medium. The correlation between the pH of the reaction medium and the ratio of the formation of **II** and **V** has been demonstrated [8]. At the optimum pH of 1.8–2.2, the enhanced reactivity of the 8-amino group in **I** leads to a high yield of **II**, whilst an increase in the pH of the reaction medium results in a decrease in the ratio of **II** to **V**. When the temperature of the reaction medium exceeds the optimum temperature (10–11°C), degradation of the diazonium salt occurs leading to a high content of the starting material (**I**) in the final preparation. Therefore it is apparent that maintenance of the potency of the bulk drug and of the relative ratios of the isomers (and, consequently, batch to batch therapeutic equivalence) is dependent on the optimisation, robustness and reproducibility of a manufacturing process with potential for significant variability.

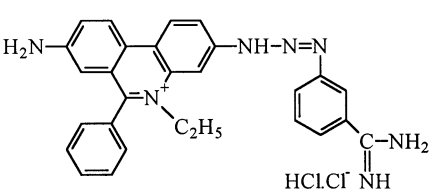
As part of a multi-faceted program of research on trypanocides, we recently developed a selective, reproducible and precise HPLC method for the determination of ISM in the presence of its related substances [9]. The current report examines products containing isometamidium from



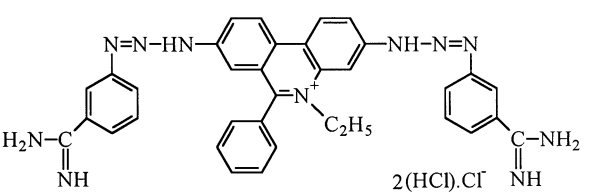
M&B 4180A (ISM, **II**)



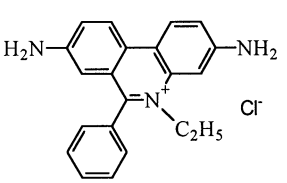
M&B 4250 (**V**)



M&B 38897 (**III**)



M&B 4596 (**IV**)



Ethidium Chloride (**I**)

Fig. 1. Chemical structures of ISM and related substances.

two different sources (Samorin®, Rhône Merieux, France and Veridium®, Sanofi Nutrition Animale, France) for their content of ISM and for the relative ratios of the principal isomers. In addition, a modification of the Berthelot or Indophenol reaction [10,11] has been developed and used to determine the content of a potential manufacturing impurity, ammonium chloride, in the batches examined.

The definitive characterisation of batches of different brands of products containing ISM by the use of chromatographic profiling, analysis of the principal component (**II**) and the relative amounts of related substances will allow more informed judgments to be made, clinically and economically, concerning the potential interchangeability of such products.

2. Experimental

2.1. Materials and reagents

Orthophosphoric acid (85% w/v), potassium dihydrogen orthophosphate and ammonium chloride ($\geq 99.5\%$ purity) were obtained from BDH Laboratory Supplies (Poole, UK). HPLC-grade methanol and acetonitrile were obtained from Merck (Lutterworth, UK). Sodium nitroprusside (sodium nitroferricyanide(III) dihydrate, 99% A.C.S. reagent) and sodium hypochlorite solution (10–15% available chlorine) were obtained from Sigma–Aldrich (Dorset, UK). Sodium salicylate was obtained from Fisons Scientific Apparatus (Loughborough, UK).

8-(3-*m*-Amidinophenyl-2-triazeno)-3-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride ((90% w/w), isometamidium, M&B4180A, batch GHS331, **II**), 3-(3-*m*-amidinophenyl-2-triazeno)-8-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (M&B38897, **III**), 7-(*m*-amidinophenyldiazo)-3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (M&B4250, **V**) and 3,8-di(3-*m*-amidinophenyltriazeno)-5-ethyl-6-phenylphenanthridinium chloride dihydrochloride (M&B4596, **IV**) were gifts from Rhône-Poulenc Rorer (Dagenham, UK). 3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide (ethidium, **I**) was a gift from Laprovet (Tours Cedex, France). Sachets (1 g) of Samorin® (Lots M157971 (expiry 05/2002), M158971 (expiry 05/2002), M159971 (expiry 05/2002) and M160971 (expiry 05/2002)) and of Veridium® (Lots 1A2 (expiry 08/2001), 2A1 (expiry 10/2001), 3A1 (expiry 04/2002) and 4A1 (expiry 06/2002)) were obtained from commercial sources.

2.1.1. Salicylate/nitroprusside reagent

Sodium salicylate (7.5 g) and sodium nitroprusside (0.15 g) were placed in a 100-ml volumetric flask and made up to volume with water.

2.1.2. Sodium hypochlorite solution (containing 0.079% w/v free chlorine)

Sodium hypochlorite solution (containing 10–15% available chlorine) was standardised by titration with sodium thiosulphate solution (0.1 M), using starch as indicator. The sodium hypochlorite solution was subsequently diluted with 0.2 M sodium hydroxide to produce a solution containing 0.079% w/v free chlorine.

2.2. Sample preparation

2.2.1. Standard solutions of ISM (batch GHS331)

For HPLC analysis, the standard sample of ISM (10 ± 1 mg) was accurately weighed using a Mettler TA5 analytical balance (readability 0.01 mg). The weighed sample was dissolved in, and made up to 100 ml with a solution of 30% v/v acetonitrile in water to produce a 0.01% w/v stock solution. Portions of the stock solution were suitably diluted with 30% v/v acetonitrile in water to produce 0.0001, 0.0002, 0.0003, 0.0004 and 0.0005% w/v solutions of ISM.

2.2.2. Sample preparation for determination of ISM

Samples (100 mg) of Samorin® and Veridium® were weighed accurately, made up to 100 ml with methanol and an aliquot (5 ml) was diluted to 100 ml with 30% v/v acetonitrile in water. The resultant solution was diluted 10-fold to produce a solution of a nominal concentration of 0.0005% w/v. Solutions of the commercial samples, were

prepared at a concentration of 0.002% w/v in 30% v/v acetonitrile in water for chromatographic profiling.

2.2.3. Sample preparation for ammonium chloride determinations

Commercial samples (0.5 g, W_{sam}) containing ISM were weighed accurately into 250-ml volumetric flasks, dissolved in and made up to volume with, water.

2.2.4. Standard solution of ammonium chloride

Ammonium chloride (0.1 g, W_{std}) was accurately weighed and placed in a 500-ml volumetric flask, dissolved in and made up to volume with, water. An aliquot (10 ml) of the resultant solution was transferred to a 100-ml flask and made up to volume with water.

2.3. Methods

2.3.1. HPLC

Samples were analysed by HPLC as previously described [9]. Chromatographic peaks were identified using authentic standards of **I**, **II**, **III**, **IV** and **V** (Fig. 1).

2.3.2. Colorimetric determination of ammonium chloride

2.3.2.1. Reference standard. Standard ammonium chloride solution (2 ml) and 3 ml of water were transferred to a 25-ml volumetric flask. Salicylate/nitroprusside reagent (5 ml) was added to the flask and the contents thoroughly mixed. An aliquot (5 ml) of sodium hypochlorite solution (containing 0.079% w/v free chlorine) was added and the resultant solution allowed to stand for 30 min after mixing.

2.3.2.2. Reagent blank. The procedure for the reference standard was followed with the use of 2 ml of water in place of the standard ammonium chloride solution.

2.3.2.3. Sample. An aliquot (1 ml) of sample solution and 4 ml of water were transferred to a 25-ml volumetric flask. Salicylate/nitroprusside reagent (5 ml) was added to the flask and the contents thoroughly mixed. An aliquot (5 ml) of sodium hypochlorite solution (containing 0.079% w/v free chlorine) was added. The resultant solution was mixed and allowed to stand for 30 min.

2.3.2.4. Sample blank. An aliquot (1 ml) of sample solution and 9 ml of water were transferred to a 25-ml volumetric flask. An aliquot (5 ml) of sodium hypochlorite solution (containing 0.079% w/v free chlorine) was added. The resultant solution was mixed and allowed to stand for 30 min. Then the reaction mixtures were made up to volume with water and thoroughly mixed. The absorbances of the resultant solutions were measured against water at 665 nm using 1-cm cells. The content of ammonium chloride (% w/w) in the sample was calculated from the following equation:

$$\%\ \text{Ammonium chloride} = \frac{(A_{sam} - A_{sb} - A_{rb}) \times W_{std} \times 10}{(A_{std} - A_{rb}) \times W_{sam}}$$

where A_{sam} and A_{std} are the absorbances of sample and standard respectively, and A_{sb} and A_{rb} are the absorbances of the sample blank and reagent blank, respectively.

3. Results and discussion

The constituents of Samorin and Veridium were baseline resolved using the previously described HPLC method [9]. Calibration curves for the analysis of ISM by HPLC were linear ($y = 10^{10}(1.03 \pm 0.09)x - 10^4(2.74 \pm 3.68)$ [mean $\pm$ S.D., $n = 3$], $r^2 = 1.000$) with low magnitudes of standard residuals randomly distributed about the trend line. ISM is stable in aqueous solutions for at least 2 h as demonstrated previously by determining the R.S.D. value for the ISM peak after replicate injections of a solution of Samorin® (R.S.D. = 0.51%, $n = 10$ [9]). In this study all solutions of ISM, Samorin® and Veridium® were freshly prepared prior to analysis. Data concerning the content of ISM in the batches of Samorin® and Veridium® analysed are given in Table 1; the results are the means of two determinations. Samorin® demonstrated a batch-to-batch consistency with respect to the content of ISM of

Table 1
Content of ISM (**II**) and ammonium chloride in different batches of Veridium® and Samorin®

Sample	ISM content (% w/w)	NH_4Cl content (% w/w)
Samorin Batch M157971	59.0	0.1
Samorin Batch M158971	60.4	0.1
Samorin Batch M159971	59.0	0.2
Samorin Batch M160971	61.7	0.2
Veridium Lot 1A2	74.5	0.9
Veridium Lot 2A1	53.4	25.8
Veridium Lot 3A1	53.6	27.1
Veridium Lot 4A1	38.6	38.9

$60.1 \pm 1.3\%$ w/w (mean $\pm$ S.D., $n = 4$). In contrast, Veridium® demonstrated considerable batch-to-batch variations with the ISM content ranging from 38.6 to 74.5% w/w ($55.0 \pm 14.8\%$ (mean $\pm$ S.D., $n = 4$)). The wide spread of values observed for the batches of Veridium® (R.S.D. = 26.8%) compared with the batches of Samorin® (R.S.D. = 2.2%) may illustrate the difficulty of controlling the manufacturing process and the concomitant implications for the composition of the product.

Chromatographic profiles obtained with 0.002% w/v solutions of samples of batches of Samorin® and Veridium® (in 30% w/v acetonitrile in water) are shown in Fig. 2. The response factors of the three major related substances (**III**, **IV** and **V**) in commercial samples were determined relative to that of ISM (**II**). A summary of relative response factors (mean $\pm$ S.D., $n = 4$) for the three major related substances in each proprietary brand of isometamidium is presented in Table 2. The relative response factors of **III**, **IV** and **V** were consistent in all the different batches ($n = 4$) of Samorin® analysed with R.S.D. of 2.7, 24.8 and 5.8% respectively. In contrast, there was marked inter-batch variation in relative response factors between the four batches of Veridium® with R.S.D. of 45.4, 85.5 and 84.7% for **III**, **IV** and **V** respectively.

In Veridium Lot 1A2, the high content of **II** (74.5% w/w) was accompanied by the absence of **V**, which was present in varying amounts in the other three batches. This finding could have been predicted from the synthetic reaction, in which the choice and control of the pH of reaction determines the relative ratios of **II** and **V** [8]. It is abundantly clear from Fig. 2 that the chromatographic profiles of the four batches of Samorin® and of Veridium® demonstrate that there are marked differences between the compositions of

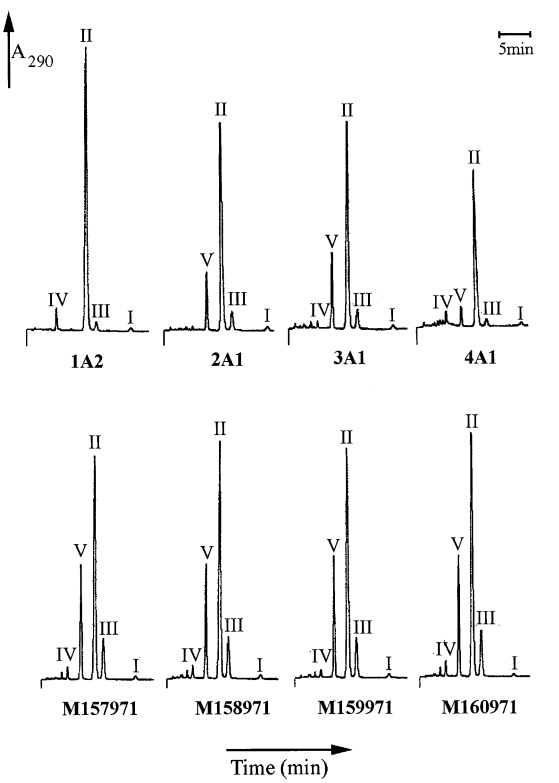


Fig. 2. HPLC profiling of ISM (**II**) and related substances (**I**, **III**, **IV** and **V**) in different batches of Samorin® and Veridium® using a 125×4 mm Lichrospher® Select-B column (mobile phase: 25% v/v acetonitrile in 20 mM phosphate buffer, adjusted to pH 3 with orthophosphoric acid). The upper and lower panels represent chromatograms obtained with 20-μl injections of 0.002% w/v solutions of Veridium® (Lot 1A2, 2A1, 3A1 and 4A1) and Samorin® (Batch M157971, M158971, M159971 and M160971) respectively.

Table 2
Relative response factors of related substances in Veridium® and Samorin®

Sample	Relative response factor (%)		
	M&B38897 (**III**)	M&B4596 (**IV**)	M&B4250 (**V**)
Samorin® Batch M157971	20.1	2.2	36.9
Samorin® Batch M158971	20.2	3.9	32.4
Samorin® Batch M159971	20.7	3.7	36.6
Samorin® Batch M160971	19.4	4.1	35.0
Veridium® Lot 1A2	3.4	4.7	0.0
Veridium® Lot 2A1	9.7	0.0	19.0
Veridium® Lot 3A1	9.5	2.1	26.3
Veridium® Lot 4A1	5.2	6.3	9.0

the batches of products made by the two manufacturers.

A rectilinear relationship ($y = (248.83 \pm 0.37)x - 10^{-2}(0.35 \pm 0.21)$ [mean $\pm$ S.D., $n = 2$], $r^2 = 1.000$) was obtained between absorbance readings at 665 nm and the content of ammonium chloride (in spiked samples of the bulk preparation) within the range 0–40% w/w. Low magnitudes of residuals were observed about the regression line. Recoveries of ammonium chloride at concentrations of 1, 10 and 30% w/w in spiked samples of Samorin® (corrected for matrix effect) were 93.2, 95.1 and 100.4% respectively. The precision of replicate determinations at a concentration of 0.2% w/w ammonium chloride in Samorin® was illustrated by a low spread of values (0.21 ± 0.03 (mean $\pm$ S.D., $n = 5$)). The content of ammonium chloride in four batches of Samorin® and Veridium® is given in Table 1. It was consistent and less than 0.30% w/w (maximum of 0.21% w/w) in the four batches of Samorin® analysed. However, the samples of Veridium® showed a wide range (0.9–38.9% w/w) for the content of ammonium chloride.

4. Conclusions

The use of chromatographic profiling, analysis of the principal component (**II**) and determination of ammonium chloride in different batches ($n = 4$) of Veridium® and of Samorin® have illustrated the implications of the complex nature and difficulty of controlling the manufacture of **II**. The innovator product, Samorin®, which has been on the market for over three decades, shows consistency (quantitatively and qualitatively) of the components in the batches analysed ($n = 4$). Batch-to-batch ($n = 4$) variations in the nature and relative response factors of related substances, content of **II**, **III**, **IV**, **V** and ammonium chloride have been demonstrated in Veridium®. The use of HPLC profiling and determination of relative response factors of related substances, coupled with determination of absolute contents of isometamidium and ammonium chloride, is a useful protocol for establishing chemical equivalence (or otherwise) between batches of an apparently similar product made by different manufacturers and between different batches of the product emanating from the same manufacturer.

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