ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 713–718

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

HPTLC and HPLC determination of isometamidium in the presence of its manufacturing and degradation impurities

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

[a] *Department of Pharmaceutical Sciences, University of Strathclyde, 204 George Street, Glasgow G1 1XW, UK*
[b] *Bioengineering Unit, University of Strathclyde, 204 George Street, Glasgow G1 1XW, UK*

Received 5 November 1997; accepted 5 November 1997

Abstract

The determination of the phenanthridine trypanocide, isometamidium chloride hydrochloride (ISM), in the presence of four process-related and degradation impurities, by RP-HPLC using a Licrospher-60 RP-select B column with a mobile phase composition of acetonitrile/KH_2PO_4 (pH 3.0, 20 mM) (25:75 v/v) with UV detection at 320 nm, is described. The method is selective, reproducible and precise with a limit of detection of 45 ng ml^{-1} for ISM. A HPTLC system (Kieselgel 60 F254, pyridine/acetonitrile/butanol/formic acid, 6:6:4:1, v/v), with UV densitometric evaluation at 320 nm, suitable for the separation of ISM and the related substances is reported.

Keywords: Isometamidium; Reversed-phase chromatography; Thin layer chromatography; Drug impurities

1. Introduction

Isometamidium (8 - [(*m* - amidinophenylazo) - amino] - 3 - amino - 5 - ethyl - 6 - phenylphenanthrid - inium chloride hydrochloride (ISM, **II**, Samorin®, Fig. 1) is the only agent used for the chemoprophylaxis of trypanosomiasis in animals in affected areas world-wide [1]. Samorin® is defined as a mixture of isomers [2,3] and its pharmacological action is due primarily to ISM [4]. No published method is available for the identification and quantitation of related substances and determination of the purity of ISM. Accurate, specific and well-characterized methods for the determination of ISM in the presence of its manufacturing and degradation products are necessary for development and quality control of the bulk manufacturing process and the assessment of stability of the bulk drug substance. In addition, the development of a specific analytical method will facilitate the interpretation of pharmacological and biological data.

Samorin is synthesized on an industrial scale by the coupling of diazotised *m*-aminobenzamidine monohydrochloride with 3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride (**I**, Fig. 1) to

* Corresponding author.

0731-7085/98/$19.00
PII S0731-7085(98)00008-9

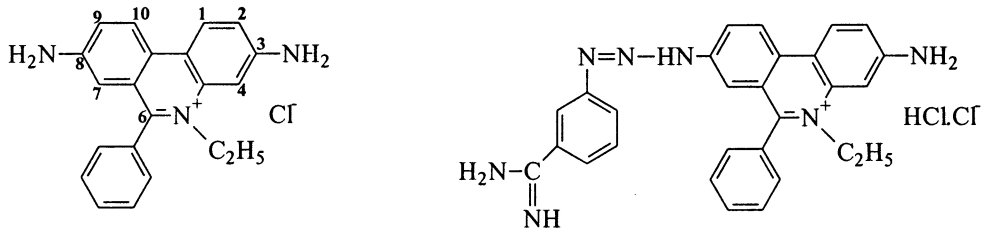

Fig. 1. Chemical structures of 3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride (**I**) and ISM (**II**).

give a mixture of isomers [2–4], with ISM as the predominantly major component. The benzene-diazonium ion is a weak electrophile, so that enhancement of reactivity by appropriate substitution with an electron-withdrawing moiety greatly extends the range of coupling [5]. The triazene linkage in ISM is acid-labile and its cleavage results in reversion to **I**, the starting material in the synthesis [6].

The presence of the quaternary nitrogen atom and strongly basic amidino moieties in ISM and the related substances presents a significant analytical challenge because of the tenacity with which such groups interact with residual silanol groups. The resultant pronounced tailing of chromatographic peaks led previous workers [7,8] to incorporate mobile phase additives leading to a reduction in the efficiency of the column. The HPLC method devised by Perschke and Vollner [7] is non-specific in that ISM is converted to **I** by acid hydrolysis, prior to quantitation. Several workers have exploited the intrinsic fluorescence of ISM in quantitative analysis [6,8,9]. However, the contribution of the related substances to the total observed fluorescence has not been evaluated.

This report describes the development of a sensitive RP-HPLC method for the determination of ISM in the presence of its known process-related and degradation impurities. In addition, the specificity of fluorescence detection in the analysis of ISM in Samorin has been investigated. A HPTLC system with UV densitometry has been developed also for the identification of ISM and related substances.

2. Experimental

2.1. Materials and reagents

HPLC-grade methanol and acetonitrile, analytical reagent grade formic acid (98% v/v), orthophosphoric acid (85% w/v) and potassium dihydrogen orthophosphate were obtained from BDH Laboratory Supplies (Poole, UK). Analytical reagent grade pyridine and butanol were obtained from Aldrich Chemicals (Dorset, UK).

(8-[(*m*-Amidinophenylazo)amino]-3-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (ISM, **II**), ISM manufacturing impurities (**III**, **IV** and **V**, purity $>85\%$) and Samorin (Lot P517) were gifts from Rhône-Poulenc Rorer (Dagenham, UK). 3,8-Diamino-5-ethyl-6-phenylphenanthridinium chloride (**I**) was a gift from Laprovet (Tours Cedex, France).

2.1.1. Sample preparation

Samples for qualitative TLC were prepared in methanol at a concentration of 0.1% w/v. Four calibration standard solutions of **II** (0.025, 0.050, 0.075, 0.100 mg ml^{-1}) in methanol were prepared for quantitative analysis.

HPLC samples were prepared in 30% v/v acetonitrile in water. Standard calibration solutions of **II** were prepared at concentrations of 0.0001, 0.0002, 0.0003, 0.0004 and 0.0005% w/v.

2.2. Apparatus

2.2.1. HPTLC with UV densitometric detection

A Shimadzu CS-9000 dual-wavelength flying

spot scanner (Shimadzu, Kyoto, Japan) was used for densitometric evaluation of developed plates with the following settings; photomode: absorbance/reflectance, λ: 320 nm, Zero set mode: at start, beam size: 0.4×0.4 mm, δy: 0.04 mm, beam swing width: 4 mm, scan-mode: zigzag, minimum spot-width detection: 0.1 mm, minimum area: 0, smoothing: 7 points, output: area, b.c. accumulation: 8, linearizer: off.

Solutions of samples were applied to Kieselgel 60 F_{254} plates (10×10 cm, 0.25 mm thickness; E. Merck, Darmstadt, Germany) using a Camag Nanomat III automatic spotter (Camag Muttenz, Germany) equipped with 1 μl capillary pipettes. Development of the plates was carried out in a Camag horizontal developing chamber (10×20 cm; using 5 min for solvent saturation of the tank) at ambient temperature. A solvent system consisting of pyridine/acetonitrile/butanol/formic acid (6:6:4:1, v/v) was used.

2.2.2. HPLC

The chromatographic system consisted of a Spectra-Physics P100 isocratic pump equipped with a Spectra-Physics SP8450 UV-Vis detector (Thermoseparations Inc., California, USA) set at 320 nm. Fluorescence detection ($\lambda_{ex} = 385$, $\lambda_{em} = 590$ nm) was performed with a Model RF-530 fluorescence detector (Shimadzu Ltd, Kyoto, Japan). Chromatograms were acquired and analysed with a HP 3395 reporting integrator (Hewlett-Packard, Waldbronn, Germany) with the following settings; attenuation: 6, peak-width detection: 0.04, threshold: 4, chart speed: 0.3 cm min^{-1}. Chromatographic separations were performed at ambient temperature with a mobile phase flow-rate of 1 ml min^{-1}. Samples were prepared in 30% v/v acetonitrile in water and injected through a fixed (20 μl) PEEK loop onto a Lichrospher® 60 RP-select B column (Hewlett Packard, Waldbronn, Germany, 125×4 mm I.D., 5 μm particle size, 60 Å) with a 20×2 mm I.D. guard column packed with Lichrosorb C8, 5 μm particle size (E. Merck, Darmstadt, Germany). The mobile phase was composed of acetonitrile/KH_2PO_4 (pH 3.0, 20 mM) (25:75 v/v). The phosphate buffer (KH_2PO_4, pH 3.0, 20 mM) was prepared by accurately weighing 1.36 g of KH_2PO_4 and making up to 500 ml with water. Then the pH of the solution was adjusted to 3.0 with orthophosphoric acid.

3. Results and discussion

Several solvent systems were evaluated in the separation of ISM and its related compounds on TLC plates coated with silica gel. The tenacity with which the phenanthridines bind to the silanol groups of silica was such they could not be eluted with single solvents like methanol. Successful planar chromatography required the presence of another ammonium ion in the mobile phase to compete with the phenanthridines for active silanol groups. However, compact spots were not obtained with solvent systems containing NH_4^+ because of the high water content. The pyridinium ion, generated in situ by the addition of formic acid and pyridine, proved to be a suitable ion to compete for silanol groups. The resultant spots were compact, well-resolved and visible under normal light, since they were brightly coloured (Table 1). Spots were identified by colour, direct comparison with authentic standards (**I**, **II**, **III**, **IV** and **V**) and reference to the published literature [3,4]. The relationship between the amount of ISM applied to the plate (x μg), and the observed densitometer response (y) was established by non-linear regression analysis using a second-order polynomial ($y = -ax^2 + bx + c$, where a and b are constants) with a mean correlation coefficient

Table 1
HPTLC data of ISM (**II**) and related substances (**I**, **III**, **IV**, **V**)

Compound	Colour	R_f
I	Red	0.71 ± 0.02
II	Orange	0.47 ± 0.02
III	Orange	0.53 ± 0.01
IV	Yellow	0.22 ± 0.01
V	Purple	0.35 ± 0.01

R_f values are mean ± S.E. mean, $n = 5$.

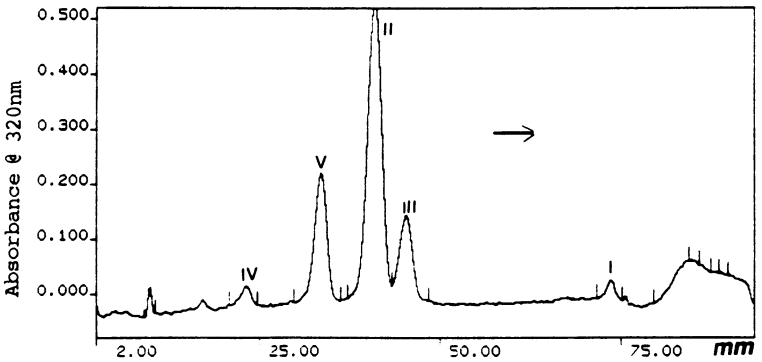

Fig. 2. Densitometric scan of a high performance thin-layer chromatogram of ISM (**II**) and its related substances. Eluent system: pyridine/acetonitrile/butanol/formic acid (6:6:4:1 v/v). Other chromatographic conditions were as described in Section 2.2.1.

(r) of 1.000 ($n = 4$). The limit of detection of ISM using the parameters described in Section 2.2.1 was 20 ng ($S/N = 3$). However, the inherent non-linearity of calibration curves for **II** excluded the use of HPTLC in routine quantitative analysis. Fig. 2 shows a densitogram of the analysis of **II** in a typical manufacturing batch (Lot P517).

Successful HPLC of the phenanthridines required the use of a base-deactivated silica-based stationery phase. Lichrospher-60 RP-select B afforded low peak tailing (asymmetry factor at one-tenth peak height $\cong 1.10$) at the maximum working concentration of **II** of 0.005% w/v. The pH of the mobile phase was maintained at 3.0 to suppress residual interactions with silanol groups. Optimum resolution of all the constituents of Samorin (Lot P517) was obtained with a mobile phase composition of acetonitrile/KH_2PO_4 (pH 3.0, 20 mM) (25:75 v/v), with $R_{s\,min}$ of 2.9 for the two isomers (**II** and **III**, $R_s = 2\ (t_2 - t_1)/(W_2 + W_1)$, where t_1 and t_2 denote the elution times of compounds **II** and **III**, and W_1 and W_2 denote the width at peak base of compounds **II** and **III**) and a $k_{max} = 13$ (compound **I**, t_0 = retention time of unretained acetonitrile). Fig. 3a shows the separation of **II** and related substances in a typical manufacturing batch of Samorin (Lot P517) monitored at 320 nm. Peaks were identified using the authentic standards (**I**, **II**, **III**, **IV** and **V**). Then the same batch of Samorin (Lot P517) was analysed using the same chromatographic system with a fluorescence detector set at $\lambda_{ex} = 385$ nm, $\lambda_{em} = 590$ nm. Fig. 3b shows a chromatogram obtained with an increased integrator sensitivity (integrator attenuation = 1); thus demonstrating a four-fold increase in sensitivity with UV detection at 320 nm.

It was apparent from these results that the fluorescence detection settings of $\lambda_{ex} = 385$ nm, $\lambda_{em} = 590$ nm [6,8] were not specific for **II**; that related substance **III** possessed a greater quantum yield of fluorescence (ϕ) than **II** and could contribute significantly in the analysis. Compound **IV** was detected at these fluorescence settings. The detector output (Fig. 3b) with fluorescence analysis, obtained with a higher sensitivity setting relative to that used in UV analysis, illustrated that UV detection afforded a superior sensitivity for **II** analysis. This is consistent with the structure of **II**, where rotational freedom of the *m*-amidinobenzenediazo substituent increases the probability that excited molecules will decay to the ground state by rotational and vibrational transfer of energy, leading to a decreasing probability of photon emission. This is supported by the observed increase in fluorescence emission of **II** in viscous solutions and after intercalation with DNA [10]. It is consistent also with reduced transfer of absorbed UV energy to emitted fluorescence in dilute solutions due to considerable internal conversion processes, like free rotation of the substituent.

Replicate injections of **II** using a sample injection loop made of stainless steel produced low precision (R.S.D. > 10%, $n = 10$). An increase in loop-flush volume with solutions of **II**, below and

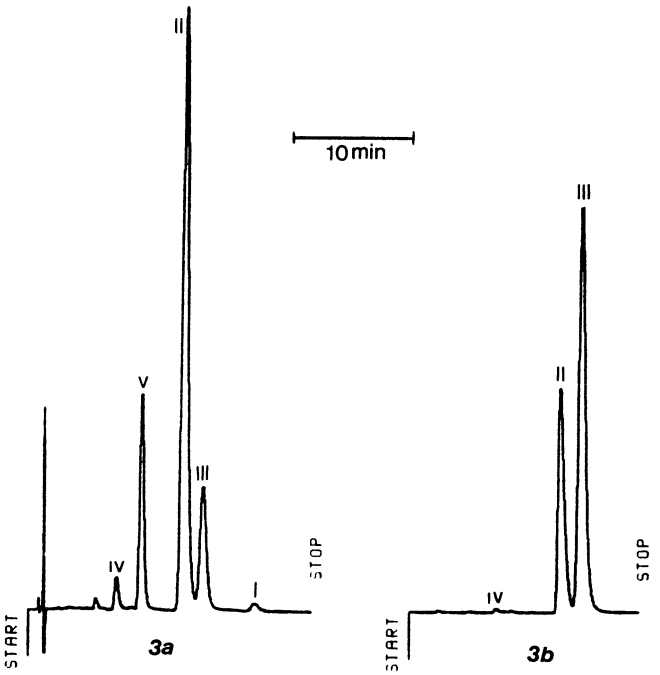

Fig. 3. High performance liquid chromatogram of ISM (**II**) and related substances in Samorin (Lot P517) using the conditions described in Section 2.2.2 with (a) UV detection at 320 nm and (b) fluorescence detection with increased integrator sensitivity (Section 3). Mobile phase: acetonitrile/KH_2PO_4 (pH 3.0, 20 mM) (25:75 v/v). Retention times (min): **IV**, 7.7; **V**, 10.1; **II**, 13.6; **III**, 15.5; **I**, 21.1.

up to the rated loop capacity (20 μl), produced a corresponding linear increase in detector response (Fig. 4). However, with sample loop-flush volumes above the rated loop capacity, a further increase in detector response was observed; a feature characteristic of sample adsorption on loop surfaces [11–13]. Peak symmetry and chromatographic resolution were not affected. The use of an injector loop made of polyetheretherketone (PEEK) improved the precision of replicate injections of solutions of **II** (R.S.D. = 0.51%, $n = 10$). The effect of increasing sample loop-flush volumes up to, and above, the rated loop volume exhibited characteristics typical of an ideal non-adsorbing loop with PEEK (Fig. 4). Calibration solutions prepared within a working range of 0.0001–0.0005% w/v **II** and encompassing the nominal assay value of 0.00025% w/v were linear ($r^2 = 0.997 \pm 0.002$ (mean ± S.D.), $n = 5$) with small standard residuals randomly distributed about the trend line.

Batches of Samorin ($n = 6$) were analysed by the HPLC method for the content of **II** in the presence of all the known impurities. Inter- and intra-day assay variation were calculated as 0.87 and 1.30%, respectively ($n = 4$). The limit of detection (three times the average noise level) of **II** was determined as 45 ng ml^{-1}.

4. Conclusion

The possible contribution of **III** and **IV** to total observed fluorescence ($\lambda_{ex} = 385$ nm, $\lambda_{em} = 590$ nm) in the spectroscopic determination of **II** in Samorin has been demonstrated. Therefore chromatographic separation of the components in Samorin prior to UV or fluorescence detection provides selectivity in the analysis of ISM (**II**). It has been demonstrated that UV detection is more sensitive than fluorescence detection in the analysis of ISM (**II**). A mobile phase (consisting of

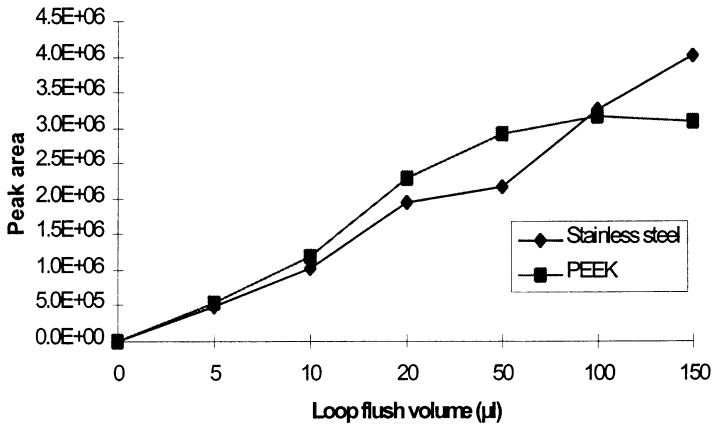

Fig. 4. Effect of increasing loop flush volume of 0.0003% w/v ISM in 30% v/v acetonitrile through injector and 20 µl loop made of stainless steel (◆) and PEEK (■). Mobile phase: acetonitrile/KH_2PO_4 (pH 3.0, 20 mM) (25:75 v/v), flow rate 1.0 ml min^{-1}. Chromatographic conditions are as stated in Section 2.2.2.

acetonitrile and phosphate buffer) with a Lichrospher-60 Select-B column resolved ISM (**II**) and its process-related and degradation impurities. The method described is simple, selective, precise, and suitable for quality control of the bulk drug substance.

HPTLC with UV densitometry using pyridine-based solvent systems provides a rapid and reproducible screen for the identification of related substances in manufacturing batches of Samorin.

Acknowledgements

We are grateful to Rhône-Poulenc Rorer, Dagenham, UK for gifts of **II**, **III**, **IV**, **V** and Samorin, and for technical support. The gift of **I** from Laprovet, France, and helpful discussions with Dr J. Wilkes (ILRI, Nairobi, Kenya) are gratefully acknowledged.

References

[1] C.C. Wang, Annu. Rev. Pharmacol. 35 (1995) 93–127.
[2] S.S. Berg, Br. Pat. Appl., 931,227 (1963).
[3] E.G.C. Clarke (Ed.), Isolation and Identification of Drugs in Pharmaceuticals, Body Fluids and Post-mortem Material, vol. 2. The Pharmaceutical Press, London, 1975, p. 1053.
[4] L.D.B. Kinabo, J.A. Bogan, J. Vet. Pharmacol. Ther. 11 (1988) 233–245.
[5] S.S. Berg, J. Chem. Soc. 1 (1963) 3635–3640.
[6] F.S. Philips, S.S. Sternberg, A.P. Cronin, J.E. Sodergreen, P.M. Vidal, Cancer Res. 27 (1967) 333–349.
[7] H. Perschke, L. Vollner, Acta Trop. 42 (1985) 209–216.
[8] L.D.B. Kinabo, J.A. Bogan, Acta Trop. 45 (1988) 165–170.
[9] D. Zilberstein, J. Wilkes, H. Hirumi, A.S. Peregrine, Biochem. J. 292 (1993) 31–35.
[10] J.M. Wilkes, A.S. Peregrine, D. Zilberstein, Biochem. J. 312 (1995) 319–329.
[11] J.W. Dolan, Liq. Chromatogr. Gas Chromatogr. 9 (1996) 530.
[12] J.W. Lough, J.M. Malcolm, J. Maltas, J. Chromatogr. 726 (1996) 67–75.
[13] S.K. MacLeod, D.T. Fagan, J.P. Scholl, J. Chromatogr. 502 (1990) 236–240.