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Evaluation of the stability of frusemide in intravenous infusions by reversed-phase high-performance liquid chromatography

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Summary

A rapid, flexible method based on ion-pair reversed-phase high-performance liquid chromatography is described for the separation and quantitation of frusemide and its principal hydrolysis product, saluamine, in the presence of potential contaminants, photolytic products and other degradants. The method is stability-indicating and has been shown to be applicable to the determination of frusemide and saluamine in injectable forms of frusemide. Response was linear both for frusemide (0–50 μg/ml; $r = 0.9999$) and saluamine (0–10 μg/ml; $r = 1.0000$). The on-column sensitivity of the assay was 1.3 ng frusemide and 1.1 ng saluamine. The method was applied to intravenous admixtures of frusemide and in studies on the effect of heat and/or light stress on other intravenous dosage forms. The saluamine assay was found to be sufficiently sensitive to monitor low levels of degradation of frusemide in the absence of photolytic degradation.

When frusemide injection was transferred to a polypropylene syringe for slow intravenous injection and stored at room temperature unprotected from light for 24 h, the level of frusemide degradation, as indicated by saluamine, remained at 0.2%, which was within the 1% compendial limit of saluamine in frusemide. When used as an additive in Compound Sodium Lactate Injection BP (Hartmann's Solution) or Sodium Chloride Injection BP 0.9% w/v, frusemide injection was stable for 24 h without protection from light. An autoclaved infusion of frusemide was stable for 10 weeks at room temperature when protected from light. A preliminary investigation of photolytic degradation of frusemide indicated a fall in pH of 3 units, precipitation

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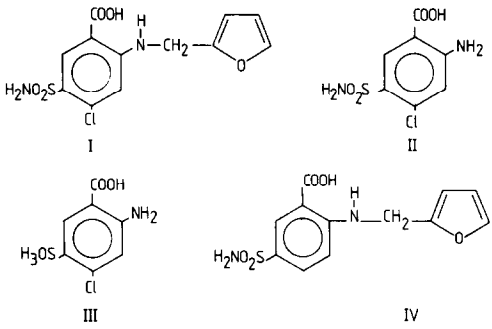
and the presence of a number of uncharacterized photolytic degradation products. A number of impurities were detected in Saluamine BP Chemical Reference Substance.

Introduction

Since current clinical practice uses intravenous infusions as vehicles for the administration of drug products, there is need for stability-indicating assays and methodology to assess stability under the conditions of administration.

Frusemide (Furosemide) Injection BP (Lasix, Hoechst U.K.) is considered to be representative of a typical additive in terms of its physical and chemical properties and was chosen for investigation on this basis. The injection is available in amber glass ampoules containing sodium frusemide equivalent to 10 mg of frusemide per ml and is prepared by the interaction of frusemide with sodium hydroxide (BP 1980 Vol. II). The product is formulated to a pH of about 9.0 and since frusemide is susceptible to oxidation, the ampoule is sealed under nitrogen. Frusemide (I) is a sparingly soluble carboxylic acid derivative which may be precipitated in acid conditions (Neil, 1976). The furfuryl group is acid-labile (Stürm et al., 1966) and frusemide is reported to hydrolyze to 4-chloro-5-sulphamoyl-anthranilic acid (saluamine) (II) (Stürm et al., 1966; Kovar, 1974; Cruz et al., 1979). The compendial limit for free amines in Frusemide Injection BP is 1.0% (BP 1980 Vol. II). I is reported to be susceptible to photodegradation and requires to be protected from light (European Pharmacopoeia, Supplement to Vol. III, 1977). Until recently the products of photochemical degradation of I in aqueous solution have been the subject of controversy. Rowbotham et al. (1976) claimed that UV irradiation of I for 48 h in alkaline solution produced 4-chloro-5-sulpho-anthranilic acid (III) by oxidation of the sulphamoyl group ($-SO_2NH_2$) to sulphonic acid with hydrolysis of



Scheme 1. Structural entities: I, frusemide; II, saluamine; III, 4-chloro-5-sulphoanthranilic acid; IV, N-furfuryl-5-sulphamoylanthranilic acid.

the furan ring. By contrast, Moore and Tamat (1980) reported complete dechlorination of I after UV irradiation of frusemide in deoxygenated neutral aqueous solutions. Since completion of the present work, Moore and Sithipitakas (1983) have reported that UV irradiation of I in methanol at 365 nm results primarily in photo-reduction to N-furfuryl-5-sulphamoyl-anthranilic acid (IV) and photohydrolysis to II (Scheme 1).

Frusemide is a loop diuretic and is given intravenously for severe refractory and pulmonary oedema associated with acute cardiac failure and also in severe acute or chronic renal failure. The injection is administered by slow intravenous (bolus) injection directly into the vein or by intermittent or continuous infusion in a suitable intravenous infusion, the addition being made extemporaneously before administration. The reported alkaline stability (Cruz et al., 1979) and the relatively stable liquid preparation (pH 8.0) described by Purkiss (1977) suggested the possibility of formulating a heat-sterilized solution of I for administration by continuous or intermittent infusion. This approach avoids the need to prepare admixtures extemporaneously, with their attendant risk of extrinsic microbial contamination (Denyer et al., 1981). However, there is consequently a requirement to assess the stability of such a heat-sterilized solution.

Pharmacopoeial methods, which control the purity of I or its content in pharmaceuticals utilize volumetric analysis (USP, 1975; European Pharmacopoeia, Supplement to Vol. III, 1977), visible spectrophotometry (USP, 1975; European Pharmacopoeia, 1977) or ultraviolet spectroscopy (USP, 1975; BP, 1980). The content of free primary aromatic amines is controlled as II by pharmacopoeial limit tests based on thin-layer chromatography (BP 1980, Vol. II) or visible spectroscopy (European Pharmacopoeia, Supplement to Vol. III, 1977). These methods lack specificity in that they are inapplicable to the assay of products exposed to light.

This is also the case with respect to other published methods for the assay of I or II in drug products or in biological fluids, which use techniques such as visible spectrophotometry (Andreasen and Jacobsen, 1974; Purkiss, 1977; Hoechst, 1979; Steiness et al., 1979), ultraviolet spectrometry (Cruz et al., 1979), spectrofluorimetry (Kindt and Schmid, 1970; Forrey et al., 1974; Dreux and Halter, 1976; Mikkelsen and Andreasen, 1977; Schäfer et al., 1977), gas chromatography (Aranda et al., 1978; Hoffmann, 1979) and high-performance liquid chromatography (HPLC) (Macdougall et al., 1975; Ghanekar et al., 1978; Lin et al., 1979; Nation et al., 1979; Smith et al., 1980; Roth et al., 1981; Rapaka et al., 1982).

Until recently little attention has been paid in published methods to possible photolytic degradation of I; only in a few cases have precautions been taken to protect standards and test substances from light. Since completion of the present work, Moore and Sithipitakas (1983) separated and identified IV and other photodecomposition products of I in oxygen-free methanolic solution using reversed-phase LC-MS and gas chromatography–mass spectrometry (GC-MS). In the LC-MS method components were resolved isocratically but analysis time was 36–40 min; this system was also used to determine the extent of photolytic degradation of I in methanol and in aqueous buffers in the pH range 9–12. The GC-MS method required prior derivatization, so that any compound which was inefficiently deriva-

tized or unstable at elevated temperature would not necessarily be detected by the system. The present work reports the development of rapid, specific and sensitive quantitative procedures for the simultaneous assay of I and II in intravenous solutions of frusemide exposed to light.

Experimental

Reagents and materials

Frusemide (4-chloro-2-furfuryl-amino-5-sulphamoylbenzoic acid) and saluamine (4-chloro-5-sulphamoyl-anthranilic acid) were generously provided by Hoechst U.K. Pharmaceuticals Division (Milton Keynes, Bucks.). Their identity was confirmed by ultraviolet, infra-red, proton magnetic resonance and mass spectroscopy. 4-Chloro-5-sulphamoyl-anthranilic acid (BP Chemical Reference Substance) was purchased from the British Pharmacopoeia Commission. HPLC grade *n*-propanol (Rathburn Chemicals, Walkerburn, U.K.) was used as received. Cetrimide was BP grade (ICI Pharmaceuticals Division, Cheshire, U.K.). All eluent mixtures were filtered through a Millipore 0.45-μm MF filter using an all-glass apparatus, before degassing for 10 min in an ultrasonic bath under reduced pressure. Sodium hydroxide and potassium dihydrogen phosphate were Analar grade (Hopkins and Williams, Chadwick Heath, U.K.). Sodium chloride was BP grade (Evans Medical, Liverpool, U.K.).

Compound Sodium Lactate Injection BP (Hartmann's Solution) packed in a 500 ml 'Viaflex' bag made of polyvinyl chloride, and sodium chloride Injection BP 0.9% packed in a 100 ml 'Viaflex' bag, were obtained from Travenol Laboratories (Thetford, U.K.), Polypropylene syringes were obtained from Becton Dickinson, (Cowley), Oxfordshire. According to information from the manufacturer (personal communication, 1984) no significant loss of water vapour occurs from Viaflex bags in 24 h. Frusemide Injection BP (10 mg/ml) was purchased from Hoechst U.K. Pharmaceuticals Division. 5-μm SAS-Hypersil was obtained from Shandon Southern Instruments (Cheshire, U.K.).

Standard solutions

Frusemide and saluamine are poorly soluble in water but are soluble in 95% ethanol or 0.01 M NaOH. For qualitative HPLC either of these solvents were used to prepare solutions of analytes. A number of solutions of I in 0.01 M NaOH were subjected to heat or light stress, or stored unprotected from light for different periods of time and examined chromatographically in order to identify and separate potential degradation products. Solutions used for qualitative development were: saluamine 0.5 mg/ml in 0.01 M NaOH (coded S); frusemide 0.5 mg/ml in 0.01 M NaOH (coded F); frusemide 0.5 mg/ml and saluamine 0.5 mg/ml in 0.01 M NaOH (coded FS); frusemide 1.0 mg/ml in 0.01 M NaOH autoclaved at 118°C for 3 h (coded FA); frusemide 1.0 mg/ml in 0.01 M NaOH irradiated at 254 nm for 8 h (coded FUV); frusemide 1.0 mg/ml stored unprotected from daylight for 48 h (coded FL); frusemide in NaOH solution stored unprotected from light for approximately 3 years (coded F3); mixture of frusemide irradiated at 254 nm for 8 h

and saluamine containing 0.33 mg/ml I and 0.17 mg/ml of II, prepared from solutions FUV and S (coded FUVS). All solutions were protected from light after preparation.

For quantitative analysis, two sets of stock solutions of I and II were freshly prepared periodically, one for the macro-level assay of I and the other for the micro-assay of II. Each stock solution was chromatographically stable for 4 days at ambient temperature (< 25°C) when stored in light-resistant containers after which they were discarded. The stock solutions contained frusemide 1.0 mg/ml in 0.05 M NaOH and saluamine 0.01 mg/ml in 0.001 M NaOH, respectively. Standard solutions of I and II for the analysis of intravenous dosage forms were freshly prepared from stock solutions by quantitative dilution immediately before use and stored in light-resistant containers, and contained frusemide 0.40 mg/ml and saluamine 0.005 mg/ml, respectively.

Equipment

The liquid chromatograph was assembled in the laboratory and comprised a liquid chromatography series 1 pump (Perkin-Elmer, Beaconsfield, UK), a variable wavelength UV-monitor with an 8-μl flow cell (Pye-Unicam, Cambridge, U.K.) and recorder type 56 (Perkin-Elmer). The 100 × 5 mm i.d. stainless-steel column (Shandon Southern Instruments, Cheshire, U.K.) was slurry-packed with a 5-μm microparticulate bonded reversed-phase packing material (SAS-Hypersil) by the upward displacement technique of Knox (1978). Sample introduction was by 20-μl Rheodyne loop valve injector (Model 7125). The chromatograph was flushed clean at the end of each day with methanol–water (40 : 60 v/v) to prevent salt deposition in the system; this flushing procedure was adopted as standard practice throughout. This eluent was also employed to check the column performance using a test mixture containing phenol and 4-cresol; the average number of theoretical plates per meter (N) was > 33,000. Test samples were heated for 3 h at 118°C in an autoclave ('Pharmacist MkIII', Surgical Equipment Supplies, London, U.K.) or irradiated at 254 nm in a thin-layer chromatography viewing cabinet for 3 h. Control solutions were stored at 25°C in a humidity cabinet ('Patra', Laboratory Thermal Equipment, Oldham, U.K.).

The following procedure was developed to reject syringe carry-over artefacts, to differentiate between analyte and solvent peaks and to detect substances present in trace amounts. Each analyte solution was injected in duplicate at a sensitivity setting of 0.2 or 0.32 AUFS using twice the volume for the second injection. The procedure was repeated at a sensitivity setting of 0.05 or 0.02 AUFS. Each solvent was treated in the same manner. Authentic peaks were differentiated from artefacts by observing the peak height, which should approximately double on injecting twice the volume of analyte or solvent.

HPLC procedure

Choice of reversed-phase HPLC. Ion-pair chromatography was chosen on account of its ability to separate ionic or ionizable molecules (Pryde and Gilbert, 1979). The reversed-phase mode was considered the most convenient because of its flexibility in

the choice and variation of mobile phase, its ability for 'fine tuning' retention (Knox and Laird, 1976) and its potential for good resolution of closely related acidic substances (Fransson et al., 1976; Knox and Laird, 1976). HPLC methods for frusemide utilizing an acidic mobile phase (Lindström 1974; Blair et al., 1975; Carr et al., 1978; Roseboom and Sorel, 1978; Broquaire and Mitchard, 1979; Swezey et al., 1979) suffer from the disadvantage that breakdown of frusemide may occur during chromatography on account of its reported acid lability; a neutral or alkaline mobile phase was therefore considered necessary. Moreover, this medium would present frusemide in an almost completely ionized form ($pK_a = 3.9$), which in turn would facilitate ion-pairing with a suitable cationic ion-pairing agent.

Preliminary experiments. Chromatography of a synthetic mixture of I and II on 5-μm ODS-silica (ODS-Hypersil) with ultraviolet detection at 229 nm with an eluent comprising *n*-propanol–20 mM KH_2PO_4 (pH 7.0) (30 : 70 v/v) containing 0.25% w/v cetrimide, at a flow-rate of 0.7 ml/min, represented optimum conditions in terms of phase capacity ratios (k′) and column efficiency (N). Under these conditions the retention time of I (25.1 min) was unacceptably high; reduction to an acceptable analysis time for I of 10.8 min using a flow-rate of 1.5 ml/min could only be achieved at the expense of decreased efficiency. Comparison of the k′ profiles for I and II suggested that dissimilar retention mechanisms were in operation. There was little variation in the k′ value of II over the cetrimide concentration range of 0.1–1.0% w/v. This indicated poor ion-pairing of the degradation product, whereas the retention of frusemide varied significantly with this parameter. The k′ value of I also decreased significantly with increase in *n*-propanol concentration; that of II was not affected.

Substitution of tetrabutyl ammonium (TBA) for cetrimide as pairing-ion and the use of *n*-propanol, methanol and acetonitrile in turn as organic modifiers gave similar results, in that acceptable analysis time of I could only be achieved at the expense of reduced resolution of II and other weakly retained polar components.

Choice of packing material. The use of the more polar short alkyl chain packing material (SAS-Hypersil) was adopted in order to improve the resolution of the more polar compounds present in the sample of saluamine, while decreasing the retention of frusemide.

Choice of detector wavelength. Operation of the UV detector at 229 nm resulted in absorptive interference from both cetrimide and TBA; a wavelength of 273 nm was therefore chosen to permit the detection of I and II without interference from the mobile phase components.

Choice of mobile phase. Initially the mobile phase used was *n*-propanol–20 mM KH_2PO_4 (pH 7.0) (25 : 75 v/v) containing 1.0% w/v cetrimide, when good separation of I ($k' = 4.4$) and II ($k' = 2.4$) was achieved. Column performance, phase capacity ratios and column efficiency were systematically optimized in terms of pH (Figs. 1 and 2), pairing-ion concentration (Figs. 3 and 4), and organic modifier (Figs. 5 and 6).

Inspection of Fig. 2 showed that both I and II exhibited their highest chromatographic efficiency at pH 7.0 (33,000 plates/m). Peak tailing was more pronounced at pH 3 and 4, which perhaps reflected different rates of elution from the column, and

Fig. 1.

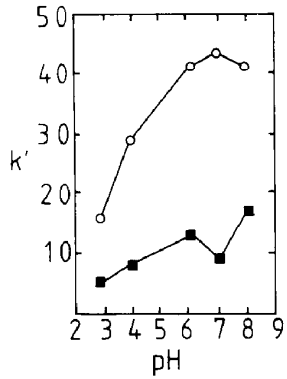


Fig. 2.

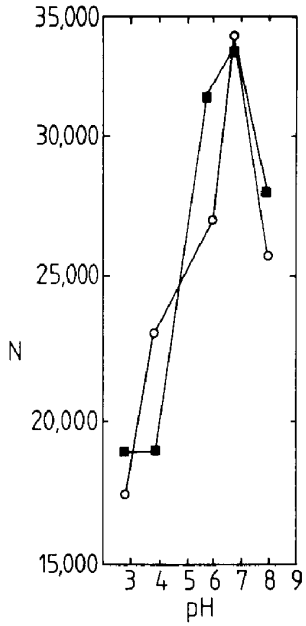


Fig. 1. Variation of k' with pH for frusemide (○) and saluamine (■).

Fig. 2. Variation of N (plates/m) with pH for frusemide (○) and saluamine (■).

Fig. 3.

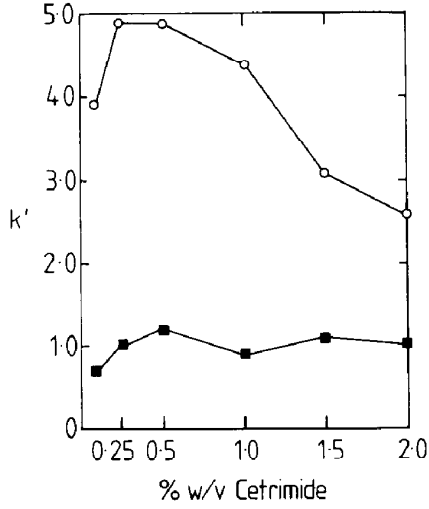


Fig. 4.

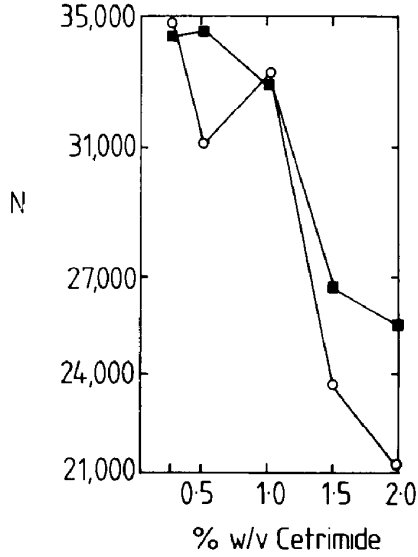


Fig. 3. Variation of k' with concentration of cetrimide (% w/v) for frusemide (○) and saluamine (■).

Fig. 4. Variation of N (plates/m) with concentration of cetrimide (% w/v) for frusemide (○) and saluamine (■).

therefore different retention of the unionized and ionized species present in approximately equal concentrations at these pH values. This may be explained on the basis that the unionized form of both compounds did not ion-pair with the cetrimide cation and as a consequence had little affinity for the lipophilic stationary phase; the unionized form therefore eluted more rapidly than the ionized form. On the other hand, the ionized form of I which predominates at pH 6–8 appeared to effectively ion-pair with cetrimide, as evidenced by the comparatively high k′ values obtained in this pH range compared to those obtained at low pH.

Further evidence of ion-pairing of I is given in Fig. 3 where the k′ value is seen to vary with cetrimide concentration. Under these conditions, however, retention of II was not affected by the cetrimide concentration, thus indicating that any ion-pairing which did occur was likely to be weak.

The classical relationship between concentration of organic modifier and retention of both I and II is illustrated in Fig. 5 where the k′ values of both compounds decreased with increase in *n*-propanol concentration.

At pH 7.0 the highest number of theoretical plates (33,000–35,000 plates/m) was obtained at a cetrimide concentration of 0.25% w/v (Fig. 4) the *n*-propanol concentration being 25% v/v (Fig. 6). This cetrimide concentration corresponded to the highest k′ value (4.9) and the most stable part of the curve of k′ versus cetrimide concentration (Fig. 3). These conditions represented optimum stability and column efficiency (n = 34,000 plates/m) and were used throughout.

Fig. 5.

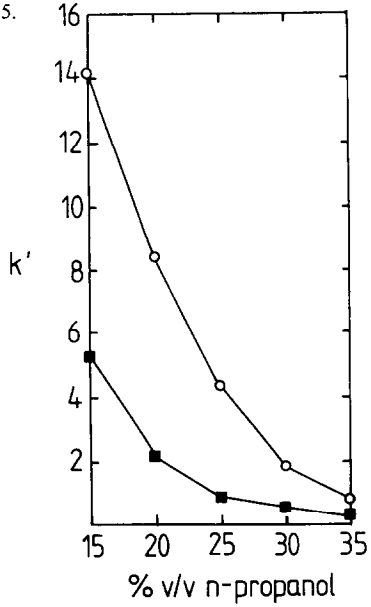


Fig. 6.

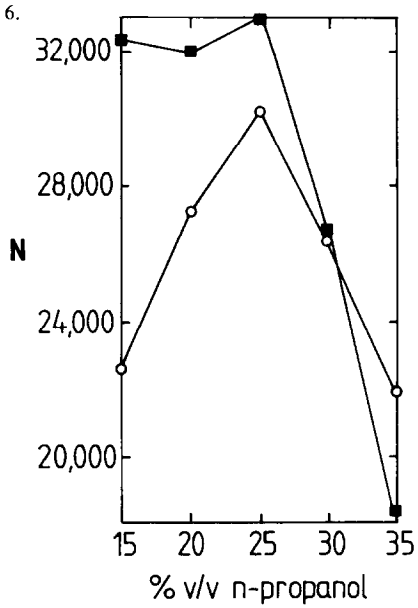


Fig. 5. Variation of k′ with *n*-propanol concentration (% w/v) for frusemide (○) and saluamine (■).

Fig. 6. Variation of N (plates/m) with *n*-propanol concentration (% v/v) for frusemide (○) and saluamine (■).

Potential contaminants and degradants. The mean phase capacity ratios of common peaks in solutions of frusemide and saluamine including those subjected to heat and/or light stress are shown in Table 1.

A liquid chromatogram of a synthetic mixture of the frusemide sample exposed to light with added saluamine is illustrated in Fig. 7; this revealed the presence of an unknown photolytic degradant as a major peak, whose k′ value corresponded with that of peak 9 (Table 1). This peak was subsequently shown to have a pronounced shoulder at a lower flow-rate (0.8 ml/min), indicating that it may represent more than one substance. The absence of this peak in the 3-year-old solution of frusemide, which had been stored unprotected from light and was coloured yellow, suggested that it was possibly due to intermediate breakdown products of photolysis.

Quantitative procedure

Choice of calibration standards for assay of I (macro assay). The most common

TABLE 1

MEAN PHASE CAPACITY RATIOS (k′) OF PEAKS IN SOLUTIONS OF FRUSEMIDE AND SALUAMINE INCLUDING FRUSEMIDE SOLUTIONS EXPOSED TO HEAT AND/OR LIGHT STRESS

Peak no.	Solution								
	S	F	FS	FA	FUV	FL	FL3	FUVS	Peak designation
1	–	1.63	1.63	1.58	1.61	1.59	1.6	1.59	Minor peak of frusemide
2	–	–	–	–	–	–	1.75 *	–	
3	2.03	2.13	2.05	2.05 *	2.15 *	2.08 *	–	2.06	Second major peak of saluamine solution S
4	2.44	2.41	2.44	2.34	2.36	2.35	2.39	2.41	Saluamine
5	–	–	–	2.73 *	2.7 *	–	2.8 *	–	Degradant of frusemide
6	–	3.0 *	–	2.93 *	2.93 *	2.88 *	3.2 *	–	
7	3.6 *	–	3.5 *	3.23 *	3.53 *	–	–	–	
8	3.95 *	3.9 *	3.83 *	3.8 *	3.9 *	3.9 *	3.98 *	3.9 *	
9	–	4.78 *	4.63 *	4.53 *	4.59	4.63	4.9 *	4.82	Photolytic degradant of frusemide
10	–	5.28 *	5.13 *	–	5.1 *	–	–	–	
11	6.43 *	6.25 *	5.98 *	5.8 *	5.9 *	5.78 *	5.6 *	6.2 *	
12	7.5 *	7.11	6.86	6.73	6.76	6.81	7.31	7.14	Frusemide
13	8.75 *	–	–	–	–	–	–	–	
14	–	–	10.26	–	–	–	–	10.6	
15	11.0 *	–	–	–	–	–	–	–	

* Observed only at 0.02 AUFS

Codes: S = saluamine, 0.5 mg/ml in 0.01 M NaOH; F = frusemide 0.5 mg/ml in 0.01 M NaOH; FS = frusemide 0.5 mg/ml and saluamine 0.5 mg/ml in 0.01 M NaOH; FA = frusemide 1.0 mg/ml in 0.01 M NaOH autoclaved at 118°C for 3 h; FUV = frusemide 1.0 mg/ml in 0.01 M NaOH irradiated at 254 nm for 8 h; FL = frusemide 1.0 mg/ml stored unprotected from daylight for 48 h; F3 = frusemide in NaOH solution stored unprotected from light for over 3 years; FUVS = mixture of frusemide irradiated at 254 nm for 8 h and saluamine containing 0.33 mg/ml I and 0.17 mg/ml of II.

concentration of I in intravenous admixtures is 1.0 mg/ml. The choice of concentration of I used as calibration standards was made so that (1 + 1) dilution of infusion admixture (test solution) would correspond with a calibration standard of 0.5 mg/ml, at a sensitivity setting of 0.32 AUFS and injection volume of 4-μl to give 80–90% full-scale deflection. Six calibration standards from 0.05 to 0.50 mg/ml were prepared by quantitative dilution from the frusemide stock solution.

Optimized chromatographic conditions. The parameters selected were: column, 5-μm SAS-Hypersil; chart speed, 10 mm/min; pump pressure, 1200 lbs./sq.in.; temperature, ambient; injection type, Rheodyne loop valve (20 μl); detector, ultraviolet, $\lambda = 273$ nm; sensitivity, 0.32 AUFS; injection volume, 4 μl; mobile phase, *n*-propanol–20 mM KH_2PO_4 (pH 7.0) (25 : 75) containing 0.25% w/v cetrimide. Although the highest chromatographic efficiency for I was observed at a flow-rate of 0.8 ml/min (Fig. 8) where the retention time (t_R) was 9.8 min, a flow-rate of 1.0

Fig. 7.

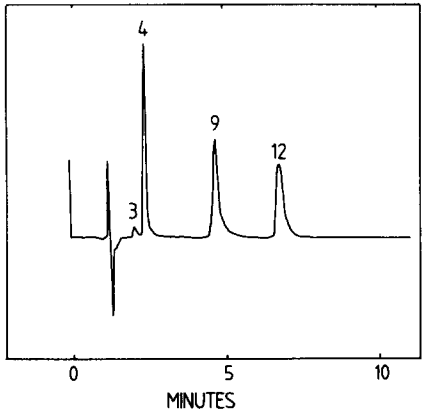


Fig. 7. Liquid chromatogram of a synthetic mixture of frusemide exposed to light with added saluamine. Chromatographic conditions as in text. Peaks: 2nd major peak of saluamine sample (3), saluamine (4), photolytic degradant of frusemide (9), frusemide (12).

Fig. 8.

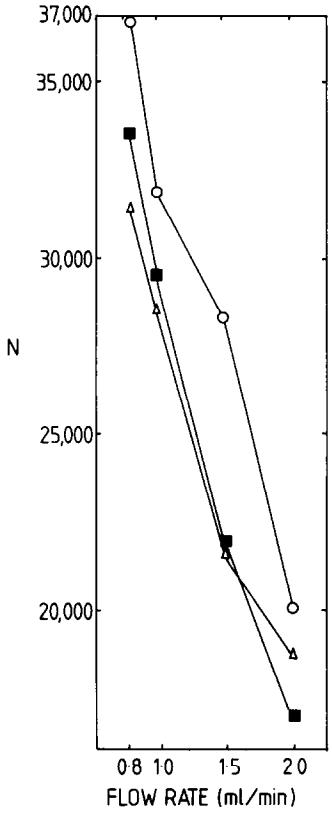


Fig. 8. Variation of N (plates/m) with flow-rate (ml/min) for frusemide (○), saluamine (■) and photolytic degradant (△).

ml/min was chosen as a compromise to achieve a more acceptable analysis time of 7.5 min.

Method of standardization. Each external calibration standard was injected in duplicate in order of increasing concentration and peak heights were subjected to regression analysis. Upper and lower 95% confidence limits for the regression line at each indicated concentration of I were calculated and expressed as a percentage of the indicated frusemide concentration.

Choice of calibration standard for micro-assay of II. The permitted limit for II in frusemide injection is 1.0% of the frusemide content (USP, 1980; BP 1980, Vol. II); the concentration of an intermediate calibration standard of II (0.005 mg/ml) in the micro-assay was chosen so that this would represent the same percentage of II with respect to the frusemide calibration standard used in the macro-assay (0.5 mg/ml). This permitted the use of the same volume and dilution (1 + 1) of infusion admixture as used in the macro-assay and allowed quantitation of II when present up to twice the limit, since instrumental conditions and injection volume of the 0.005 mg/ml standard for II (8 μl at 0.02 AUFS) were adjusted to achieve 50% deflection. Six calibration standards from 0.001 to 0.01 mg/ml of II were prepared by quantitative dilution from the stock solution of II.

Optimized chromatographic conditions were as for the macro-assay with the exception of detector sensitivity (0.02 AUFS), flow-rate (0.8 ml/min) and injection volume (8 μl). The same procedure and treatment of results were applied as in the macro-assay.

Analytical curves of peak height against analyte concentration were rectilinear and passed through or close to the origin, the relevant statistical data being summarized in Table 2. These results together with the low values of relative standard error of slope for both the macro- and micro-assay indicated analytically acceptable linearity. The single-point bracketting method was adopted in each case for routine quantitative measurements. In this technique, a group of test samples is preceded and followed by injections of a standard of comparable concentration, the average of the standards being used to calculate the test concentrations (cf. Table 2).

Reference to Fig. 9, which illustrates 95% confidence limits for macro- and micro-calibration graphs, shows that the highest relative precision was observed

TABLE 2

STATISTICAL DATA FOR ANALYSIS OF FRUSEMIDE AND SALUAMINE

Compound	Regression	Standard error of intercept (Sc)	95% confidence limits of intercept ($P = 0.05$, n = 6)	Standard error of slope (Sb)	Correlation coefficient (r)
I *	$y = 43.9x + 0.075$	0.046	± 0.113	0.156	0.9999
II **	$y = 2000x - 0.065$	0.029	± 0.071	4.93	0.9999

* Macro-assay.

** Micro-assay.

towards the top of the calibration graph in each case. At the concentrations used for assay (0.40 mg/ml for I and 0.005 mg/ml for II), the precision was considered to be analytically acceptable (relative confidence limits ($P = 0.95$) of 0.96 and 1.0%, respectively).

Standardization of saluamine sample. The saluamine reference sample (Hoechst) was freshly prepared as a solution containing 0.0122 mg/ml (equivalent to the highest calibration standard) and standardized against a solution containing 0.010 mg/ml of saluamine BP chemical reference substance (BPCRS British Pharmacopoeia Commission). The saluamine in each case was dissolved in and made up to volume with 0.001 M NaOH. Chromatography conditions were as for the micro-assay. The purity of the Hoechst reference sample was found to be 90.2% w/w with respect to saluamine BPCRS, a value which was used to correct all concentrations based on the Hoechst reference sample.

Limits of detection of frusemide and saluamine. These were determined and expressed as the weight of material injected onto the column to give a peak height equivalent to twice the base-line noise at 0.01 AUFS. For this purpose, solutions of frusemide and saluamine BPCRS (containing 0.0066 mg/ml frusemide and 0.002 mg/ml saluamine, respectively) were injected onto the column in turn, and the volume progressively reduced until the peak height was approximately 10 times greater than the peak-to-peak base-line noise.

The peak heights of two replicate injections were measured and the limit of detection for frusemide and saluamine BPCRS found to be 1.3 and 1.1 ng on-col-

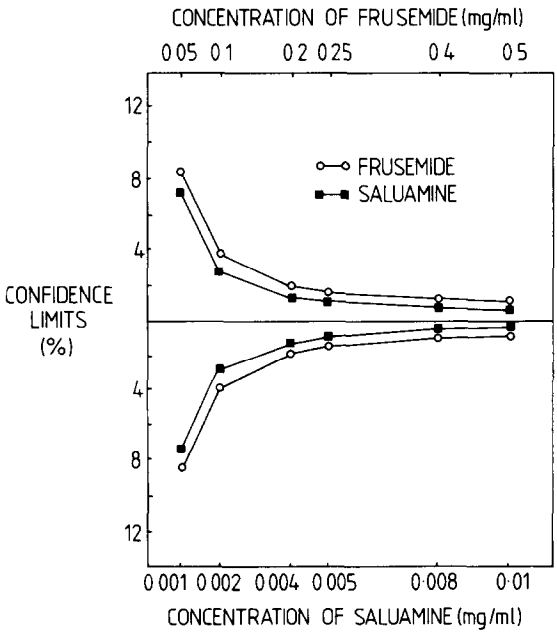


Fig. 9. 95% confidence limits for frusemide (○) and saluamine (■) calibration graphs.

umn, respectively; using an injection volume of 20 μl, these values corresponded to limits of 66 and 53 ng/ml, respectively.

Stability studies

The aim of these studies was to examine: (1) the stability of Frusemide Injection BP under conditions which simulate direct slow injection into a vein using a syringe or syringe pump; (2) the stability of extemporaneously-prepared infusion mixtures of Frusemide Injection BP in both Compound Sodium Lactate Injection BP (Hartmann's Solution) and Sodium Chloride Injection BP 0.9% w/v; and (3) the possibility of formulating a ready-made heat-sterilized infusion of frusemide and to assess its stability.

Frusemide Injection BP (Hoechst, batch 097829), of nominal content 10 mg/ml frusemide was assayed and found to contain 9.8 mg/ml I and 0.015 mg/ml II (in terms of saluamine BPCRS). This was used for stability studies (1) and (2).

Frusemide by direct slow injection. Eight 25-ml polypropylene syringes were filled with the injection and a sterile protective polypropylene cap was placed on each syringe nozzle. The syringes were divided into two groups, coded A and B. Syringes in group A were stored unprotected from light at room temperature ($< 25°C$) under normal conditions of artificial light near a window, to simulate ward conditions. Syringes in group B, which were used as a control, were stored in the dark in a hot-air oven which was maintained at $25 \pm 0.5°C$. 3-ml aliquots were pooled from the syringes in each group and were assayed for I and II after 2.5, 5 and 24 h. Each pooled sample was assayed for I and II as soon as possible after collection. All pooled samples were stored in light-resistant containers at 4°C before assay.

Hartmann's Solution mixture. 50 ml was withdrawn by syringe and needle from each of 2×500 ml bags of Hartmann's Solution and retained for pH measurement. 50 ml of the injection of I was added to each bag and thoroughly mixed; 10 ml was withdrawn from each bag and retained for pH measurement. The bags were coded HL and HC; HL was stored unprotected from light at room temperature ($< 25°C$) under normal conditions of artificial light near a window to simulate ward conditions; whereas HC was stored as a control in the dark in a hot-air oven maintained at $25 \pm 0.5°C$. 20-ml aliquots were withdrawn from each bag immediately after mixing and again after 6 and 24 h. Each aliquot was assayed for I and II as soon as possible after collection. All samples were stored in light-resistant containers at 4°C before assay.

Sodium chloride mixture. 10 ml was withdrawn by syringe and needle from each of 2×100 ml bag of Sodium Chloride Injection BP 0.9% w/v and retained for pH measurement. 10 ml of the injection was added to each bag and thoroughly mixed; 10 ml was withdrawn from each and retained for pH measurement. The bags were coded SL and SC; SL was stored unprotected from light at room temperature ($< 25°C$) under normal conditions of artificial light near a window to simulate ward conditions; SC was stored as a control in the dark in a hot-air oven maintained at $25 \pm 0.5°C$. 20 ml aliquots were withdrawn from each bag immediately after mixing

and after 24 h. Each aliquot was assayed for I and II as soon as possible after collection. The mixture which had been unprotected from light for 24 h was then stored in a refrigerator at 6°C for a further 26 days, examined for precipitation and assayed for I and II content using a 20 ml aliquot as before. The pH of this solution was also recorded. All samples were stored in light-resistant containers at 4°C before assay.

Autoclaved infusion of frusemide. I is liable to precipitate in acid solution, so that in order to prevent this, Frusemide Injection (Hoechst) should not be added to infusions which have a pH less than 5.5 (Hoechst U.K., 1979); similarly, any formulated infusion of I requires to be adjusted to a pH which prevents precipitation of the free acid. The latter was calculated from: $5.5 + \Delta pH$, where ΔpH represents the mean change in pH brought about by the addition of Frusemide Injection to Injection Sodium Chloride 0.9% w/v, to give a final concentration of 1.0 mg/ml I (5 replicates); this was found to be 7.45. The infusion was prepared by dissolving 0.25 g Frusemide BP in 0.05 M NaOH to which a solution of 2.25 g Sodium Chloride BP in 100 ml of distilled water was added and thoroughly mixed. The pH of the infusion was adjusted to nominal pH 7.45 by the addition of 0.05 M NaOH before making up to 250 ml with distilled water.

A 20 ml aliquot of the infusion (coded a) was taken for assay of I and II immediately after preparation. The remainder was used to distribute 50 ml into each of four 100-ml MRC glass infusion bottles, which were fitted with rubber bungs and capped with aluminium caps. These were divided into two groups, A and B, and individual bottles were coded b, c, d, e. Samples b, c, d and e were autoclaved at 115°C for 34 min. Samples b and c were assayed for I and II as soon as possible after sterilization and cooling. A 20 ml aliquot was taken from each for pH measurement. Sample d was unprotected from light; sample e was protected from light by covering with aluminium foil. Both were stored at room temperature and assayed for I and II content after 70 days. The mean pH of unautoclaved infusion was 7.7; after autoclaving the mean pH value was 6.6.

Results and Discussion

Reproducibility of peak height of frusemide and saluamine standards. During assay development reproducibility of instrumental response was determined at both macro- and micro- levels by injecting 10 replicates of both frusemide and saluamine standards, when the RSD values for peak heights were 1.16% and 1.48%, respectively. On application of the macro-assay to stability studies, the RSD of peak heights of frusemide standard used for the assay of I at each time interval was found to vary unacceptably. However, this was found to be attributable to an unusually high temperature coefficient for the assay, as discussed below. By the use of concurrent standards and careful attention to column temperature, RSD values of 1.3% could be attained for I and 1.0–3.9% for the hydrolysis product.

Effect of temperature on peak height and k′ of I. The variation in peak height of frusemide standard was considered to be due to room temperature variation. In a

separate experiment the peak height of I was found to increase markedly with increase in room temperature when the RSD was 14.5% over the temperature range 16.5–21.5°C. As illustrated in Fig. 10, the logarithm of k′ was seen to vary inversely with room temperature.

From these results it was evident that temperature had a profound effect on equilibrium and that control of temperature for the macro-assay was critical. The results reflected the influence on k′ normally associated with increase in temperature in excess of ambient where plots of log k′ vs $1/T$ (K^{-1}) are generally linear (Schmit et al., 1971; Snyder and Kirkland, 1979). These effects are more pronounced in ion-pairing systems than in other modes of chromatography, due to a more pronounced reduction in eluent viscosity and consequent improved mass transfer between mobile and stationary phases (Schmit et al., 1971; Snyder and Kirkland, 1979), which would suggest further confirmation of ion-pairing of I.

The RSD of peak height of saluamine standard (3.6%) obtained when the room temperature was constant at 22°C was comparable with that obtained during assay development (1.48%) and application of the micro-assay to stability studies (1.0–3.9%); this reduced response to temperature would perhaps confirm a different separatory mechanism in respect of II.

Stability of frusemide injection by direct slow injection. Inspection of the frusemide assay results (Table 3) showed that there was no detectable change in frusemide content of the injection on transfer from ampoules to syringes and upon storage unprotected or protected from light after 2.5, 5 and 24 h. The peak of the photolytic degradant (peak 9, Fig. 7 and Table 1) was only observed in the saluamine assay chromatograms; it was small and constant in the control solution (OC) and in each of the A and B solutions. This, together with the absence of colour in these solutions, indicated that no photolytic degradation had occurred and that the micro-assay could be used as a monitor of the extent of frusemide degradation under these conditions.

Results with respect to the latter showed that the injection in the original container exhibited 0.18% degradation and that this level was maintained after 24 h storage in a syringe at 25°C in the dark. On transfer to a syringe and storage unprotected from light at < 25°C for 24 h, the extent of degradation increased from 0.18% to 0.21%; this 24-h level of saluamine (0.0135 mg/ml) corresponded to 0.14%

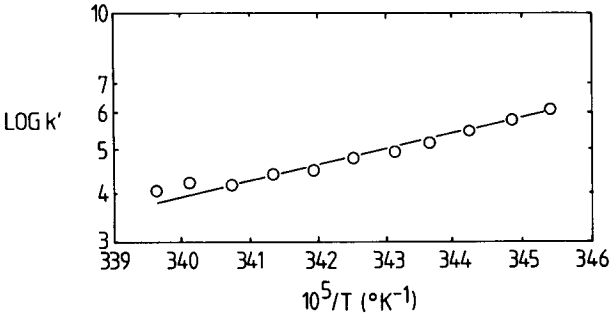


Fig. 10. Variation of retention of frusemide (log k′) with room temperature, $10^5/T$ ($°K^{-1}$).

of the mean frusemide content and was well within the compendial limit of 1.0%. It was concluded that under both sets of conditions the injection maintained acceptable stability.

Stability of Hartmann's Solution mixture. It was evident from Table 3 that there was no change in the frusemide content or extent of degradation of frusemide (as determined by the micro-assay) in the mixture stored at room temperature unprotected from light. The photolytic degradant peak 9 was only observed at 0.02 AUFS as a small peak; its height increased slightly over the 24-hour period, which suggested the possibility of incipient photolysis in this solution. No change was observed in the frusemide content of the mixture stored at 25°C in the dark. The photolytic degradation peak observed at 0.02 AUFS remained small and constant throughout the 24 h. The extent of degradation of frusemide increased from 0.19% to 0.25% in 24 h; this level (0.00185 mg/ml saluamine) corresponded to 0.20% of the mean frusemide content and was well within the compendial limit of 1.0%. The assay

TABLE 3

FRUSEMIDE AND SALUAMINE (AS SALUAMINE BPCRS) CONTENT OF FRUSEMIDE INJECTION WHEN ADMINISTERED INTRAVENOUSLY BY DIFFERENT METHODS

	Time (h)	Frusemide content (mg/ml)	total degradation (%)
Direct Injection			
OC	0	9.22–10.39	0.175–0.188
A	2.5	9.59–10.11	0.188–0.202
	5.0	9.69– 9.94	0.203–0.208
	24.0	9.34–10.32	0.202–0.215
B	2.5	9.81–10.33	0.175–0.188
	5.0	9.68– 9.94	0.171–0.176
	24.0	9.48–10.47	0.175–0.188
In Hartmann's Solution (nominally 1.0 mg/ml)			
HL	0	0.86– 0.97	0.204–0.219
	6	0.89– 0.98	0.202–0.206
	24	0.87– 0.97	0.198–0.214
HC	0	0.86– 0.99	0.183–0.198
	6	0.87– 0.96	0.195–0.199
	24	0.86– 0.96	0.235–0.254
In Sodium Chloride Injection 0.9%			
SL	0	0.96– 1.0	0.308–0.315
	24 h	0.95– 1.0	0.300–0.311
SL2	26 days	0.86– 0.89	0.301–0.307
SC	0	0.95– 0.99	0.321–0.328
	24 h	0.90– 0.97	0.282–0.292

OC = stored in original container; A = stored in polypropylene syringes at room temperature (< 25°C) unprotected from light; B = stored in polypropylene syringes at 25°C in dark; HL, SL = stored unprotected from light at room temperature (< 25°C); HC, SC = stored at 25° in the dark; S2L = stored at 6° in the dark.

results therefore reflected good stability of the admixture under the conditions of test. The greater degradation (0.25%) in the admixture stored in the dark at 25°C at 24 h compared to that found at the same time interval in the admixture stored at room temperature (0.21%) indicated that hydrolysis of I occurred at the higher temperature.

Stability of sodium chloride mixture. From Table 3 it was apparent that there was no detectable change in the frusemide content of both SL and SC after 24 h; the photolytic degradant peak 9 was small and constant in the chromatograms of both. The extent of degradation of the solution exposed to light, as measured by micro-assay, remained constant over this period; the control solution on the other hand showed a decrease from 0.33 to 0.29% (corresponding to a decrease in saluamine content of 11.6%), which may have indicated loss of II due to precipitation or adsorption on to the polyvinylchloride bag.

The frusemide content of the solution stored at 6°C for 26 days showed a mean loss of 0.88 mg/ml I (9.7%) compared to the content at 24 h; this decrease was not reflected by the micro-assay, which showed no change in saluamine content. The photolytic degradant peak 9 which appeared as a small peak at 0.02 AUFS showed a marginal increase in height. The decrease in frusemide content of the refrigerated solution without corresponding increase in II was difficult to explain. It may have been due to either precipitation of I or adsorption of this compound on to the polyvinylchloride bag or both. Precipitation was a possibility, since the pH of the isotonic saline used (4.51) was less than recommended by the manufacturer for this diluent (> 5.5), despite the fact that there was no visual evidence of a precipitate.

The overall results suggested that the admixture stored under both sets of conditions was stable for 24 h; the stability of the refrigerated solution was less clear and requires further study with the inclusion of a control.

Autoclaved infusion of frusemide. Inspection of Table 4 shows that there was no detectable change in the frusemide content of the infusion after autoclaving or on subsequent storage for 70 days at room temperature when protected from light. The

TABLE 4

CONTENT OF FRUSEMIDE AND SALUAMINE (AS SALUAMINE BP CHEMICAL REFERENCE SUBSTANCE) IN AUTOCLAVED INFUSION OF FRUSEMIDE (NOMINALLY 1.0 mg/ml) UNDER VARIOUS CONDITIONS

	Frusemide content (mg/ml)	Saluamine content (mg/ml)
a	0.96–1.08	0.00135
b	0.97–1.08	0.00235
c	0.97–1.08	0.00235
d *	0.39–0.41	0.0113
e	0.96–0.98	0.00270

* Supernatant. a = immediately before autoclaving; b, c = immediately after autoclaving; d = stored at room temperature and unprotected from light for 70 days after autoclaving; and e = stored at room temperature and protected from light for 70 days after autoclaving.

saluamine content increased from 0.00135 to 0.00235 mg/ml (i.e. by 74.1%) on autoclaving. A further increase to 0.00270 mg/ml (14.9%) was apparent in the solution protected from light after storage for 70 days at room temperature. The latter level corresponded to 0.3% w/w of the mean frusemide content of this solution and was therefore well within the compendial limit of 1.0%. There was no evidence of the photolytic degradant peak 9 in any of these chromatograms. These results indicated that the frusemide infusion was stable on autoclaving and on subsequent storage protected from light for 70 days at room temperature.

When saluamine concentration was used as a measure of the amount of degradation of frusemide, it was found that the frusemide content of the infusion on autoclaving decreased by 0.13% with corresponding decrease in pH of 1.1 units (pH 7.7 to 6.6). The extent of degradation, calculated in similar fashion, of the autoclaved infusion stored protected from light for 70 days was 0.04%. The pH of the infusion during this period was constant at 6.6–6.8. These results indicated a much slower rate of degradation of I than was found by Ghanekar et al. (1978), when a solution of I in sodium hydroxide lost 0.5% I in 30 days and by Purkiss (1977); extrapolation of the results obtained by the latter at pH 8.0 and 20°C indicated that 0.04% degradation of I occurred in 15.6 h.

Preliminary examination of photolytic degradation. During the course of storage for 70 days unprotected from light, the autoclaved infusion formed a yellow-orange precipitate. The supernatant contained 0.39–0.41 mg/ml I (Table 4); this represented a decrease of 38.8% when compared with the frusemide content of the freshly autoclaved infusion b. By contrast, the saluamine content of the supernatant, which

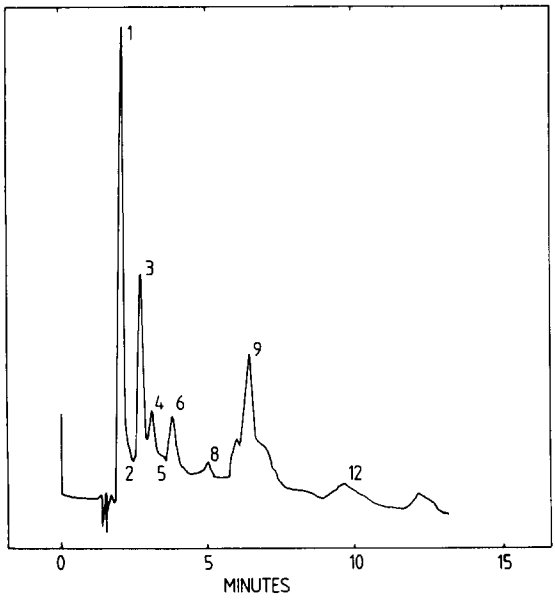


Fig. 11. Liquid chromatogram of a (1 + 1) dilution of the supernatant of infusion of frusemide which precipitated during storage when unprotected from light for 70 days: 0.02 AUFS.

was 0.0113 mg/ml (Table 4), showed an approximate 4-fold increase (3.8) when compared to b. Exposure to light and change in pH appeared to be factors in the reaction; the pH decreased from 6.6 in b to 3.42 in d, whereas, as reported previously, there was no change in pH and little change in the concentration of frusemide or saluamine in the infusion protected from light over the 70 days.

A chromatogram of 1 : 2 dilution of the supernatant at 0.02 AUFS is shown in Fig. 11. An injection volume of 1 μl was used. Peaks which corresponded with peaks given in Table 1 on the basis of their k′ values were assigned the same peak number as in Table 1. When this chromatogram was compared with the corresponding chromatogram of e, it was evident that there was a very large increase in the peak heights of peaks 1, 3, 6 and 8 as well as that of saluamine (peak 4) which suggested that these products were photolabile, particularly those represented by peaks 1 and 3, which showed the greatest increase in height. In addition, peak 9, the photolytic degradant peak previously identified, was observed as a broad large peak, with a pronounced shoulder on each side.

Inspection of a chromatogram of the precipitate from d, previously dissolved in 0.02 M NaOH, in which 1 μl was injected at 0.02 AUFS, revealed the presence of a previously unidentified peak, ($k' = 4.81$) which formed a broad double peak overlapping the frusemide peak ($k' = 4.67$). The peak area of the double peak was much less than that of the frusemide peak in the corresponding chromatogram of e; this together with the reduced frusemide content of the supernatant of e indicated considerable loss of frusemide in the autoclaved infusion stored unprotected from light for 70 days.

The chromatogram of the redissolved precipitate also revealed the presence of two other major peaks corresponding to peaks 3 and 9 previously identified in the chromatograms of the supernatant (Fig. 11). Peaks corresponding to peaks 1, 4, 5, 6 and 8 (Fig. 11) were also present.

Saluamine BPCRS. Chromatography of the sample of this material revealed the presence of 3 minor peaks ($k' = 0.47$, 1.82 and 2.94) in addition to the saluamine peak. These minor peaks appeared to correspond to peaks 2 ($k' = 0.42$), 7 ($k' = 1.77$) and the photolytic degradant peak 9 ($k' = 2.78$) listed in Table 1. The BPCRS reference material was therefore considered to be impure. A similar finding has been reported by Rapaka et al. (1982) with respect to the USP chemical reference standard for this compound.

Conclusions

The proposed assay is rapid and suitable for the separation and quantitation of frusemide and saluamine in the presence of their potential contaminants and degradants. It is stability-indicating and has been shown to be applicable to the determination of frusemide and saluamine in intravenous admixtures of frusemide and other intravenous dosage forms of frusemide which have been subjected to thermal and/or light stress. In the absence of photolytic degradation, the saluamine assay has been shown to be a sensitive monitor of frusemide degradation. The

presence of at least 7 contaminant or degradant peaks has been identified, of which 5 (peaks 1, 3, 6, 8 and 9; Fig. 11) appeared to be products of photolysis of frusemide. The method would therefore seem to be suitable for further investigation of this mode of degradation. Although frusemide and saluamine are determined separately, both assays utilize the same mobile phase; the separate chromatographic conditions required for each assay are easily achieved by alteration of the detector sensitivity and eluent flow-rate. The proposed saluamine assay is comparable with that developed by Rapaka et al. (1982), in that it can be used as a pharmacopoeial limit test for this substance in injection of Frusemide BP; it has the advantage over his method that frusemide can be assayed using the same mobile phase and the same dilution of test solution.

The sensitivity to frusemide is comparable with that of the majority of published HPLC methods; it is, however, less sensitive than that achieved by Broquaire and Mitchard (1979) and Smith et al. (1980). Despite the satisfactory reproducibility in instrumental response which was achieved during development of the frusemide assay, when it was assumed that room temperature and therefore column and mobile phase temperature was reasonably constant, the subsequent temperature dependence of peak height and consequent variation in reproducibility indicates that temperature control of the column and eluent is essential for the macro-assay.

The results from the study on stability of frusemide in injections and infusions indicate that frusemide maintains acceptable stability without protection from light for 24 h when stored in polypropylene syringes. There is therefore, no need to protect the injection from light when it is given by direct slow injection under the conditions of normal clinical use. This was also the case with respect to the extemporaneously prepared admixture of frusemide in Hartmann's Solution.

The extemporaneously prepared admixture of frusemide in isotonic saline showed similar stability. However, the use of a Sodium Chloride Injection of pH less than 5.5 and the inconclusive results with respect to the nature of the loss of frusemide in the refrigerated solution, and particularly the potential for precipitation, would suggest that further work on the stability of this mixture should be undertaken. Hartmann's Solution, therefore, would seem to be preferable as a vehicle for frusemide injection on account of its suitable pH (> 5.5) and relatively high buffer capacity due to the presence of lactate ion (Landersjö, 1978).

Frusemide maintained stability on autoclaving. The ready-prepared heat-sterilized infusion of frusemide, when stored protected from light, also showed good stability. These studies illustrate the general applicability of stability-indicating assays based on HPLC for the development and evaluation of intravenous admixture formulations in clinical pharmacy.

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