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Review

Chromatographic methods for determining the identity, strength and purity of ranitidine hydrochloride both in the drug substance and its dosage forms — an exercise in method selection, development, definition and validation

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Abstract: The selection, development, definition and validation of selective stability-indicating procedures for high-performance liquid chromatographic and thin-layer chromatographic analyses of ranitidine hydrochloride are described. The procedures used in conjunction can be applied to the quality assurance and stability assessments of both the drug substance and its dosage forms and serve to establish the identity, strength and purity of this drug used in the treatment of peptic ulcer and related conditions.

Keywords: *Ranitidine hydrochloride; drug substance; dosage forms; high-performance liquid chromatography; thin-layer chromatography; method selection; method development; method validation; related substances.*

Introduction

The impracticability of conducting toxicological assessments and clinical evaluations of each batch of a medicinal presentation as it is produced has the consequence that demonstration of the continued efficacy and safety of the preparation as supplied to the market becomes fundamentally an analytical matter, dependent upon two basic factors. These factors are: first, the true pertinence of the specifications that have been adopted; second, the calibre of the analytical methods being applied in the specification testing. These analytical methods must be capable, at established levels of accuracy, precision and sensitivity, of revealing variations within the limits set in the registered specifications and all deviations from them, should they occur, in order to establish clear criteria by which batches can be adjudged to pass or fail standards of safety, efficacy and quality.

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A basic requirement inherent in specifications testing, is that there should be proven ability of the methods to reveal any negation of the conclusions relating to the efficacy and safety of the dosage form, as drawn from the results of toxicological and clinical studies undertaken during drug development, upon which regulatory permission to market was based. In particular, the analytical methods must ensure that there are no changes in the identities, and quantities of any drug-related impurities from those present throughout the safety studies. Being closely related chemically to the active drug substance, such substances are themselves likely to exhibit biological activity which could be of an injurious nature.

Drug contamination by low concentrations of related substances is probably inevitable, not only because of the practical limitations on securing bulk chemical substances in a state of complete purity, but also because absolute chemical stability is very rarely encountered, especially in the case of organic compounds.

Changes in the profile of drug-related impurities can occur in several ways: changes in the route of synthesis of the drug substance; changes in the qualities of the starting materials, intermediates, reagents and solvents employed; and changes in the conditions used for the reactions and in the work-up and isolation procedures, within a given synthesis. These can all alter the identities and quantities of the impurities of synthesis. Similarly the identities and quantities of drug-derived degradation products can change with changes in e.g. the various constituents admixed with the drug in the dosage form; the degree of protection against the influences of light, moisture and oxygen by packaging; and the temperatures to which the pharmaceutical presentation is exposed during manufacture, storage, distribution, dispensing and use.

Thus in order to ensure the efficacy and safety of a drug, it is necessary to define specifications and methods of specifications testing. These should include the ability to identify and quantitate the drug-related substances which may safely be permitted to be present. Furthermore, the methods, together with criteria to ensure their control, must be clearly defined so as to ensure capability for reliable and reproducible performance when used in different laboratories.

Chromatography, capable of both separations and quantitative determinations, is well suited to the development of highly selective methods for active drug assay and for the determination of drug-related substances. This is especially true where two different chromatographic systems are used in conjunction so as to secure what must be mutually compatible results (with increase in related substances matched by fall in content of the drug) where both methods are operating correctly. Such a combination permits highly selective stability indicating quantitations, as well as reliable identifications, since correspondence of behaviour on two distinct chromatographic systems represents a more definitive criterion of identity than reliance upon properties such as u.v. absorption or reactions in colour tests. These properties, while characteristic of the presence of a given chromophore or chemical functionality, are never compound specific.

Moreover, since the first development and definitive validations of selective analytical methods are normally conducted in the context of a given synthetic process for the drug substance and of particular formulations of the dosage form, safety is enhanced if official methods of specification testing employ two different selective chromatographic methods, such as reversed-phase high-performance liquid chromatography (HPLC) for assay of content of the active principle, and normal phase thin-layer chromatography (TLC) for the determination of drug-related impurities. It is unlikely that both chromatographic methods will suffer equivalent interferences upon a change in the

identities of related impurities. Thus the dual application of the two procedures should reveal the presence of any previously unencountered drug-related substances which may be present in a generic product, for example, where a different synthesis has been employed for the drug substance and/or a different formulation for the pharmaceutical presentation. Recognition of such an occurrence permits work to be initiated, so as to identify the novel impurities with the appropriate necessary toxicological studies that should be undertaken.

In order to demonstrate that chromatographic methods developed for application to a particular drug, manufactured to a defined and well controlled synthesis, or to a particular dosage form of that drug manufactured to a defined formulation, are truly selective and sensitive and can provide reliable comparative information on batch to batch quality, it is necessary to have available authentic samples of all drug-related substances that may potentially be present as contaminants. These compounds include all likely major impurities of synthesis and all potential major products of any degradation which could occur during manufacture, or develop under normal storage conditions of the drug substance or its dosage forms. These compounds can then be used in challenge experiments to confirm the selectivity and sensitivity of the methods. Modern analytical method validation thus includes methods of securing such compounds by, e.g. direct isolation from mother liquors, performance of accelerated degradative experiments or independent syntheses. Description of such work is thus properly accommodated in definition of analytical methods, their development and validation, as is a description of the full characterisation of the analytical reference standard upon which quantitative chromatography, being a comparative method, is critically dependent.

Proper systematic evaluation of analytical methods to demonstrate that they are scientifically sound, is necessary to ensure that, when employed under appropriately defined conditions, they are capable of objective application in different laboratories to give consistent and reliable results with minimal inter-laboratory variability. Definition of specific application conditions for analytical methods should also be reinforced through setting limits on parameters which monitor the performance of the methods, preferably as they are being applied during actual determinations [1]. For chromatographic procedures, these so-called "system suitability limits" may be imposed on parameters such as resolution factors, retention times, column efficiency (for example, through definition of numbers of theoretical plates), peak shape (for example through definition of tailing factors) and relative standard deviations (RSDs) over a defined number of replicate determinations; and through use of standards at loadings that reflect limits of detection and limits of determination.

As is well documented [2], full method validation will normally involve demonstration of acceptable selectivity, accuracy, precision under conditions of repeatability, precision under conditions of reproducibility, sensitivity and robustness. Accuracy may be assessed by determination of the linearity of response and of the correlation coefficient, through determination of the components of total error [3] and, in the case of dosage forms, by recovery experiments. As defined in the British Standard [4], precision under conditions of repeatability involves assessment of the variability of the test results, obtained by a given method to identical test material under the same conditions (same sample solution, analyst, and apparatus in the same laboratory), over a relatively short time interval. In contrast, precision under conditions of reproducibility involves assessment of the variability among the test results, obtained with the same method on

identical test material under different conditions (different sample solutions, analysts, apparatus and laboratories), over a relatively prolonged time interval. Both assessments involve series of replicate determinations.

The present paper reports the validation of well defined chromatographic procedures suitable for the determination of the identity, strength and purity of ranitidine hydrochloride [the hydrochloride salt of compound (1) in Table 1], both in the drug substance and in the dosage forms of this selective histamine H_2 receptor antagonist. It is used in the treatment of peptic ulcers [5], reflux oesophagitis [6] and dyspepsia [7]. These methods are as submitted to the United States Pharmacopeial Convention [8].

When applied under the defined conditions, the methods provide reliable and reproducible results. There is no recourse to independently determined corrections for interferences or cumbersome sample pretreatments, as would be required for the achievement of true selectivity with a number of methods for the determination of ranitidine in dosage forms or in body fluids. These include applications of non-aqueous titrimetry [9], polarography [10], colorimetric determinations of the products formed on interaction of ranitidine with *para*-dimethylaminobenzaldehyde [11], phenylhydrazine [12] or bromothymol blue [13], the use of ion-selective electrodes [14], spectrophotometry [15–18], liquid chromatographic procedures applied to pharmaceuticals [15, 19], plasma [20–29], urine [21, 23, 28, 30], biochemical preparations [31], and liquid chromatography combined with mass spectrometry (LC/MS) [32–34] (which like radioimmunoassay [35, 36]) has been applied to assays in tissues and biological fluids. None of these methods are reported as having been validated to the extent necessary to meet current regulatory and compendial requirements with respect to drug substances and dosage forms.

When applied in conjunction, the two chromatographic methods described may be employed (together with an appropriate test to confirm the identity of any anion present) in confirming the identity of ranitidine samples and its salts. The criterion is that the sample will exhibit identical behaviour to that of the reference material on both systems.

Method selection and development for ranitidine hydrochloride

At the outset, the decision was taken to employ two distinctly different chromatographic procedures within the methods for specifications testing. These methods have been developed for ranitidine hydrochloride and its formulated products. They ensure independent but mutually cross-informative assessments of identity, strength and purity. Further, it was intended that each of the developed chromatographic methods would be capable of adaptation (with only minor modifications) to both the drug substance and the full range of dosage forms planned for the market. This would avoid inherent complications where different methods are applied to a drug substance and pharmaceutical presentations manufactured from it.

In particular, it was decided to employ reversed-phase HPLC with external standardisation using single-point peak area ratio determinations and u.v. detection for the assay of ranitidine content (i.e. strength). Normal phase TLC with two-point bracketing and visualisation with iodine vapour was chosen for the determination of ranitidine-related substances (i.e. purity). Such a choice could be seen to have a number of advantages. Thus within the assay there would be no concern over any related substances which were either eluted with the solvent front or were strongly retained on the column. Also, the HPLC method, being confined to the determination of ranitidine

content would not be complicated by the need to secure resolutions other than those between the ranitidine peak and peaks present arising either from a related substance or a constituent of a dosage form which might give a response in the chromatogram. There would also be no need to establish limits of detection for related impurities or their response factors on the HPLC system. Determination of resolutions among individual impurities and limits of detection and response factors for these impurities would be confined to the TLC method which can reveal any related substances which migrate with the solvent front or remain at the origin.

It was also decided, for both chromatographic methods, to incorporate flexibility in the preparation of solutions by use of defined loading weights. This allows for the use of different volumes of solutions of different concentrations thus allowing for situations where different laboratories might be equipped with balances and volumetric glassware of different operating ranges.

The chemical identities of the compounds required to challenge both chromatographic systems for their requisite selectivity are shown in Table 1. Compounds 3–7, which are products of hydrolysis of ranitidine [37], and compound 2 [38] were prepared as earlier described. The methods of preparation of compounds 8 and 9 are given in the experimental section of the present communication.

The primary analytical reference standard of ranitidine hydrochloride was prepared in high purity as described in the experimental section and fully characterised. Its spectroscopic properties have been reported earlier [39]. Analytical Working Standards (AWS), taken from suitable batches of ranitidine hydrochloride, were then calibrated at regular intervals against the primary standard for potency, and used in day to day laboratory work. This minimised demands on the primary standard and obviated the need for constant removal of samples of the primary standard with attendant risk of its accelerated deterioration.

The HPLC assay system

In addition to securing an HPLC system applicable to the drug substance and the various dosage forms, a simple isocratic system which can operate at ambient temperature was sought. This system would be free from the complexities of gradient elution, use of ion-pairing reagents and need for additional organic modifiers.

Development of the system, within the time available, was aimed towards optimising operating conditions by identifying a combination of stationary phase, mobile phase, column loading, flow rate and detector setting which would reliably provide high quality chromatography within an acceptable run time.

External standardisation was chosen to avoid the demands associated with the selection of a suitable internal standard. These include: ready availability; similar distribution characteristics to those of the analyte, so that equivalent proportions to those of the analyte are taken through any sample preparation; full resolutions on the chromatographic system from all potential impurities that could be present in the analyte; production of a peak shape analogous to that of the analyte; similar detector response to that given by the analyte; stability in the system, a linear concentration/response relationship and possible need to change the internal standard on a formulation to formulation basis due to specific interferences. The high precision of modern fixed volume injection loops, both manual and automated, ensures highly reproducible injection volumes and makes external standardisation a reliable method in the concentration ranges involved. This is especially the case when there is adequate

Table 1
Ranitidine and related compounds employed in the challenges for chromatographic selectivity

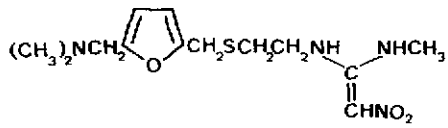
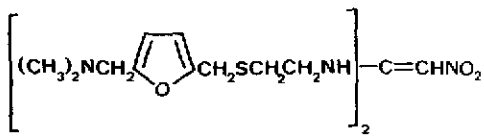
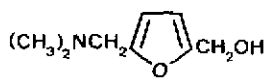
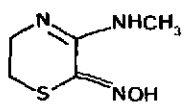
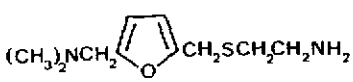
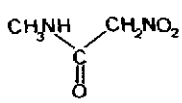
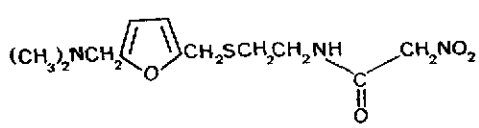
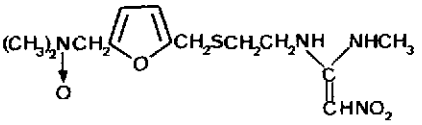
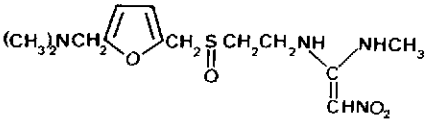
Compound number	Structure	Potential source (S = synthesis D = by degradation)
1	 Ranitidine	—
2	 *N*,*N*′-bis[2-[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]-2-nitro-1,1-ethenediamine	S
3	 5-[(dimethylamino)methyl]-2-furanmethanol	S,D
4	 5,6-dihydro-3-methylamino-2H-1,4-thiazin-2-one oxime	S,D
5	 5-[[(2-aminoethyl)thio]methyl]-*N*,*N*-dimethyl-2-furanmethanamine	S,D
6	 *N*-methyl-2-nitroacetamide	D
7	 *N*-[2-[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]-2-nitroacetamide	D

Table 1
Continued

Compound number	Structure	Potential source (S = synthesis D = by degradation)
8	 *N*-[2-[[[5-[(dimethylamino)methyl]-2-furanyl] thio]ethyl]-*N'*-methyl-2-nitro-2,2-ethenediamine *N*-oxide	D
9	 *N*-[2-[[[5-[(dimethylamino)methyl]-2-furanyl] methyl]sulphinyl]ethyl]-*N'*-methyl-2-nitro-1,1-ethenediamine	D

interspersion of standard solutions among sample solutions so as to monitor any instrumental drift.

Ranitidine hydrochloride exhibits two absorption maxima, i.e. at about 229 and 320 nm. The exact values depend on the solvent. The higher wavelength, where the absorption is attributable to the substituted 1,1-diamino-2-nitroethene portion of the molecule, was chosen for the detector setting for two reasons. First, being removed from sharply ascending or descending portions of the absorption curve, it would minimise the effect of minor errors in setting the detector wavelength. Second, it could be expected to minimise potential risk with future dosage forms of interferences within the procedure, since few excipients would be expected to absorb at this wavelength, should there be a co-elution with ranitidine.

The highly polar nature of ranitidine hydrochloride, as reflected in its very high solubility in water, suggested the use of a reversed-phase HPLC system. This would avoid unduly strong adsorption on to the stationary phase, as would be expected with a normal phase system. Unduly prolonged run times with peak broadening were expected to be avoidable with a reversed-phase system used with a mobile phase with an appropriately high polarity.

An octadecylsilylated silica (ODS) stationary phase was chosen because of its ready availability and familiarity with the use of this material. To secure a mobile phase of appropriately high polarity, methanol–water mixtures were investigated with buffering capacity by the addition of ammonium acetate, since a mobile phase of this nature could be expected to lend itself to relatively straight forward optimisation in terms of its most favourable composition without recourse to complex strategies [40].

The TLC system for determining ranitidine-related impurities

A normal phase system was sought to complement the reversed-phase HPLC method adopted for the assay and attention was directed towards chromatoplates homogeneously coated with silica particles.

For the *de novo* development of the TLC system for determination of ranitidine related substances, an eluotropic series of solvents [41–44], reflective of ability to desorb material from given adsorbents, and a microcircular technique [45–47] were used in the preliminary evaluations. This work revealed the need for a strongly alkaline mobile phase so as to eliminate tailing and to secure good migration (R_f 0.4–0.6) of ranitidine. This would provide space in front of and behind the ranitidine spot for potential related compounds of higher and lower R_f values. It further indicated that a suitable mobile phase could be made from ethyl acetate, isopropanol, water and ammonia solution.

For the developed method for the specifications testing, commercially available silica gel $60F_{254}$ chromatoplates were chosen because of their consistent quality, giving a highly reproducible performance. Here the proportions of the constituents of the mobile phase were refined until the optimal proportions were identified.

Selection of iodine vapour as the means of visualisation avoided problems associated with the markedly different u.v. absorption characteristics of some of the related substances potentially capable of being present, e.g. products of hydrolytic degradation [37].

In order to monitor the identity of the major impurity of synthesis as that present throughout the acute and chronic toxicity studies, so as to ensure safety, application of this compound (compound 2 in Table 1) as a reference standard of identity was incorporated within the TLC method.

Experimental

Materials and methods

Reagents for HPLC. All reagents were chromatographic grade. The mobile phase was a filtered and degassed mixture of methanol and 0.1 M aqueous ammonium acetate (7.71 g l^{-1}) in varying ratios. Reference materials were: (a) ranitidine hydrochloride. Analytical Working Standards (AWS) fully calibrated for potency against the Primary Reference Standard. All batches of AWS used throughout had a purity >99.5%; (b) ranitidine-S-oxide (9) as resolution standard.

HPLC instrumentation. A range of different equipments was used in the studies. This in itself is a test of the robustness of the method. The equipments included: (a) autosamplers, Perkin–Elmer LC420 and Gilson 231/401 sample injector/diluter; (b) pumps, LDC/Milton Roy Constametric IIIG, Milton Roy CM4000 and Waters 6000A; (c) detectors, Kratos Analytical Spectroflow 757, LDC Milton Roy Spectromonitor D and Pye Unicam LC-UV3; (d) integrated system, Hewlett–Packard HP 1090M incorporating photodiode array UV-Vis detector with a 4.5-μl flow cell; (e) data system, Hewlett–Packard HP 3350A and HP 3357.

HPLC column selection. This was from stainless steel tubes (200 or 250 × 4.6 mm i.d.) packed with a stationary phase of ODS bonded to 10-μm silica particles. Both Spherisorb ODS and Partisil ODS of the type optionally designated "1" were used. During the course of development, systematic variations were made in chromatographic

conditions to enable limits to be assigned to five different parameters which would ensure suitability of the HPLC system. The parameters chosen, with respect to the peak from ranitidine hydrochloride were: (a) the resolution, R_s, from the peak given by ranitidine-S-oxide (9); (b) the number of theoretical plates, N; (c) the tailing factor, f; (d) the retention time t_r; (e) the RSD over eight replicate determinations.

Solutions for HPLC. These were made in the mobile phase to given concentrations to accommodate the use of balances and volumetric glassware having different ranges of operating characteristics. In all cases, solutions were prepared to accurately known concentrations which permitted the use of different injection volumes between 1 and 100 μl.

HPLC standard solutions. With the aid of appropriate volumetric glassware, solutions of ranitidine hydrochloride analytical working standard in the mobile phase were prepared (at least in duplicate) to a precision of ±0.5%. This way, a known weight of ranitidine hydrochloride (about 1 μg) would be introduced on to the chromatographic column in an injection volume of 1–100 μl.

HPLC resolution check solution. Solutions were accurately prepared to contain known quantities of ranitidine-S-oxide (9) resolution standard and ranitidine hydrochloride working standard in the mobile phase.

HPLC sample solutions. Solutions of ranitidine hydrochloride samples were accurately prepared, at least in duplicate, by dissolution of the sample of drug substance or through extraction from, or dilution of, dosage forms. This was performed in the mobile phase to the same nominal concentration and tolerances as for the standard solutions above. These were injected in the same volume as that used for the corresponding standard solutions.

Quantitation by HPLC. Detection was normally by u.v. absorption at 322 nm. For each set of determinations, a fixed injection volume of mobile phase in the range of 1–100 μl was chosen, within which was a target for application to the column. This was normally about 1 μg of ranitidine hydrochloride, but a range of concentrations was used. The chosen volume was applied to the column via the corresponding fixed volume injection loop, in a series of single or duplicate determinations, with respect to the sample solutions. Standard and sample solutions were applied to the column in varying sequential patterns, but in general, not more than six sample solutions were applied consecutively before application of a standard. Peak areas of both standards and samples were measured and calculations were made using the mean response factor, $\bar{R}$, for the standards, as described in "Definition of the HPLC assay procedure". The ranitidine hydrochloride in the samples was thus quantitated.

Reagents for TLC. All reagents were chromatographic grade. The reference materials were: (a) ranitidine hydrochloride, AWS as defined for the HPLC method; (b) 5-[[(2-aminoethyl)thio]methyl]-*N*,*N*-dimethyl-2-furanmethanamine hemifumarate resolution standard [the hemifumarate of the trivially designated "diamine" (5)]; (c) *N*,*N*′-bis[2-[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]-2-nitro-1,1-ethenediamine identity reference standard [the trivially designated "bis compound", (2)] which was the major related substance present throughout the safety and efficacy studies.

TLC chromatoplates. Silica gel, 60 F_{254}, 0.25 mm thick, typically 20 × 20 cm. Coated plates from E. Merck Darmstadt were used without activation.

Solutions for TLC. As with the solutions for HPLC, these were prepared to given concentrations to permit the use of balances and volumetric glassware having different ranges of operating characteristics.

TLC standard solution. This was freshly prepared to various concentrations, but normally to contain about 0.0223% m/v of ranitidine hydrochloride AWS in methanol.

TLC resolution check solution. This was prepared as a solution of the hemifumarate of 5-[[(2-aminoethyl)thio]methyl]-*N*,*N*-dimethyl-2-furanmethanamine (5) in methanol and applied on top of a sample application made to the plate.

TLC related impurity identity check solution. This was prepared as a solution of *N*,*N'*-bis[2-[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]-2-nitro-1,1-ethenediamine (2) in methanol.

Sample solutions. These were made in methanol to various concentrations.

TLC conditions. Microcircular chromatography was used in the preliminary studies and an alkaline mobile phase of ethyl acetate, isopropanol, ammonia and water was identified as being suitable. Chromatography was conducted at ambient temperature using 20 × 20 cm silica gel $60F_{254}$ chromatoplates in sealed glass tanks, lined with chromatographic paper and pre-equilibrated with vapours of the mobile phase. Standards were applied at levels of 0.05–2% of the nominal sample loading, which was normally 223 μg of ranitidine hydrochloride. The diamine resolution check solution was applied on top of an application of a sample solution. Detection was by means of iodine vapour and quantitation by two-point bracketing by standards.

Syntheses

Preparation of specially purified ranitidine hydrochloride as primary reference standard. Ranitidine (20 kg) was re-crystallised twice from methylisobutylketone (2 × 133 l) to give material 14.5 kg (72.5%), m.p. 74°C. A portion (170 g) was dissolved in ethanol and filtered through a column of Kieselgel 60 (70–230 mesh ASTM). Ethanol was used as eluent to remove a trace impurity that remained adsorbed on the silica. Evaporation of the solvent and crystallisation of the residue from ethyl acetate (5 vol) gave the base as a white solid, (138 g), m.p. 74°C.

A portion of the above base (90 g) was dissolved in isopropanol AR (630 ml) at 50°C and treated with concentrated hydrochloric acid AR (23.3 ml). The mixture was stirred at 50°C for 20 min, allowed to cool to room temperature and filtered. The product was washed with isopropanol AR (100 ml) and dried to constant weight at 40°C to give white crystals (93 g), m.p. 141–142°C.

The product was assayed for ranitidine hydrochloride content by potentiometric titration with 0.1 M NaOH using a glass electrode and a silver/silver chloride reference electrode. It was found to be 100.2%. Loss on drying over phosphorus pentoxide at 60°C *in vacuo* was 0.04% m/m. The loss was entirely due to water. This was shown by the total absence of any organic solvents detectable by gas chromatography. There was no

detectable sulphated ash. Only one related substance (compound 2) was detectable by the defined TLC procedure; it was present at a level of 0.05% m/m.

The primary reference standard was then used to calibrate secondary "working standards", i.e. batches of ranitidine hydrochloride for use as subsidiary standards in day-to-day laboratory work.

Preparation of authentic samples of ranitidine-related compounds. Compounds 2–7 were obtained as described in the literature [37, 38].

N-[2-[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]-*N'*-methyl-2-nitro-1, 1-ethenediamine-*N*-oxide (8) was synthesised in three stages: (i) 5-[(dimethylamino) methyl]-2-furanmethanol *N*-oxide. 85% metachloroperbenzoic acid (38 g) was added to a solution of 5-[(dimethylamino)methyl]-2-furanmethanol (31 g) [48] in dichloromethane (400 ml) at 0°C over a period of 1 h. After stirring at 0°C for 1 h, a further 1.7 g of 85% metachloroperbenzoic acid was added and the resulting solution was allowed to stand at 0°C for 18 h. The solution was evaporated to dryness to yield a gum which was re-dissolved in methanol (200 ml) and passed through an Amberlite ion-exchange column. The methanolic solution from the column was evaporated to dryness and the resultant gum triturated with dry tetrahydrofuran to yield a pale yellow hygroscopic solid (31.6 g), m.p. 130°C which was identified by IR and ^{1}H NMR spectroscopy as 5-[(dimethylamino) methyl]-2-furanmethanol-*N*-oxide.

(ii) 5-[[(2-Aminoethyl)thio]methyl]-*N*,*N*-dimethyl-2-furanmethanamine-*N*-oxide. Small portions of 5-[(dimethylamino)methyl]-2-furanmethanol-*N*-oxide (17.1 g) were added to a stirred solution of cysteamine hydrochloride (12 g) in concentrated hydrochloric acid (40 ml) over a period of 1 h. The resulting solution was stored at 0°C for 36 h and then heated at 50°C for 1 day. The mixture was cooled to 0°C and slowly heated with an excess of sodium bicarbonate to give a friable solid. The solid was extracted with ethyl acetate (2 × 50 ml) followed by isopropanol (3 × 50 ml). The isopropanol extracts were bulked and evaporated to dryness to yield an oily residue. The residue was dissolved in chloroform (150 ml), filtered and the filtrate evaporated to dryness to yield a hygroscopic oil (23 g) which was identified by IR and ^{1}H NMR spectroscopy as 5-[[(2-aminoethyl)thio]methyl]-*N*,*N*-dimethyl-2-furanmethanamine-*N*-oxide.

(iii) *N*-[2-[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]-*N'*-methyl-2-nitro-1,1-ethenediamine-*N*-oxide (8). A mixture of 5-[[(2-aminoethyl)thio]methyl]-*N*,*N*-dimethyl-2-furanmethanamine-*N*-oxide (230 mg) and 1,1-bis (methylthio)-2-nitroethene (165 mg) in 25 ml of ethanol was heated under reflux for 16 h. The resulting mixture was treated with 33% (v/v) ethanolic methylamine (2 ml) and heated under reflux for a further 20 h. The resulting mixture was evaporated to dryness and purified by preparative liquid chromatography (silica/2% methanolic ammonia) to give a pale brown oil (79 mg). The oil was identified as compound 8 by ^{1}H NMR and IR spectroscopy and microanalysis. Compound 8 ($C_{13}H_{22}N_4O_4S$) requires: C,47.26; H,6.71; N,16.96. Found: C,47.43; H,7.06; N,16.94%.

N-[2-[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]sulphinyl]ethyl]-*N'*-methyl-2-nitro-1,1-ethenediamine (9). A solution of ranitidine hydrochloride (1.05 g) in water (25 ml) containing sodium periodate (0.71 g) was kept at 0°C for 36 h. The solution was saturated with potassium carbonate and extracted with ethyl acetate (12 × 30 ml). The ethyl acetate extracts were dried over magnesium sulphate and evaporated to dryness to yield a yellow oil. The oil was crystallised from hot ethyl acetate to give a fawn crystalline

solid (0.59 g) m.p. 136–136.5°C, identified as compound 9 by ^{1}H NMR and IR spectroscopy and microanalysis. Compound 9 ($C_{13}H_{22}N_4O_4S$) requires: C,47.27; H,6.67; N,16.97. Found: C,47.04; H,6.58; N,16.88%.

Results and Discussion

The HPLC assay method

From experiments employing a variety of concentrations of ranitidine hydrochloride AWS in mobile phase and a variety of injection volumes of these solutions, some of which were also used to determine linearity of response, it was found that satisfactory chromatography resulted from loadings spanning a wide range either side of the 1 μg target loading decided upon for the method.

The results of linearity assessments over a set of four experiments obtained with the working standard of ranitidine hydrochloride and employing injection volumes spanning those recommended for use in the method (namely 1, 10, 25 and 100 μl) are given in Table 2, together with the equations for the least-squares regression line, the intercept and the correlation coefficients. In all cases the value of the intercept is expressed as a percentage of the area value which would be given by a standard at the concentration giving a 1 μg loading.

In order to demonstrate the selectivity of the method, challenge experiments were conducted both at the fixed wavelength of 322 nm for detection and over the range 210–360 nm using a photodiode array detector, through co-chromatography of authentic samples of compounds 2–9 in Table 1 with ranitidine hydrochloride, both one at a time and in combination. In each experiment resolutions between peaks were determined.

The individual experiments involving co-chromatography of ranitidine hydrochloride AWS with each of the ranitidine related substances shown in Table 1, one at a time, revealed that the two compounds having the closest retention times to ranitidine were the two oxidation products, ranitidine-*N*-oxide (8) and ranitidine-S-oxide (9). Ranitidine-S-oxide (9) was adopted as a standard to act as an indicator that a given column is achieving adequate resolution performance.

The resolutions attained in the present study are depicted in Table 3 and Fig. 1. Table 3 lists the retention times, relative to that of ranitidine, of the related substances on the two stationary phases employed. It also shows the approximate λ_{max} of each compound in solution in mobile phase. Figure 1 shows one particular orientation of the chromatogram obtained from an artificially prepared mixture of compounds 1–9 employing a diode array u.v. detector. From Fig. 1 it can be seen that whereas compounds 2–9 are all well separated from ranitidine (1), thus conferring the required selectivity upon the HPLC system for the assay of ranitidine, compounds 3 and 4 are not fully resolved from each other. Neither compound 3 nor 4 shows absorption at the defined detector wavelength of 322 nm.

The results of assessment of the stability of ranitidine hydrochloride solutions in the mobile phase, revealed an RSD of 0.2% for the determinations on each of three solutions as made at time of preparation and after storage for 7 days, protected from light and at ambient temperatures. These results demonstrate a stability which will permit automated assay procedures with several days' run times.

The results (expressed as peak areas) obtained from the evaluation of the precision of the chromatographic stage of the method, employing 10 replicate injections of each of four standard solutions of concentrations such that injection volumes of 1, 10, 50 and

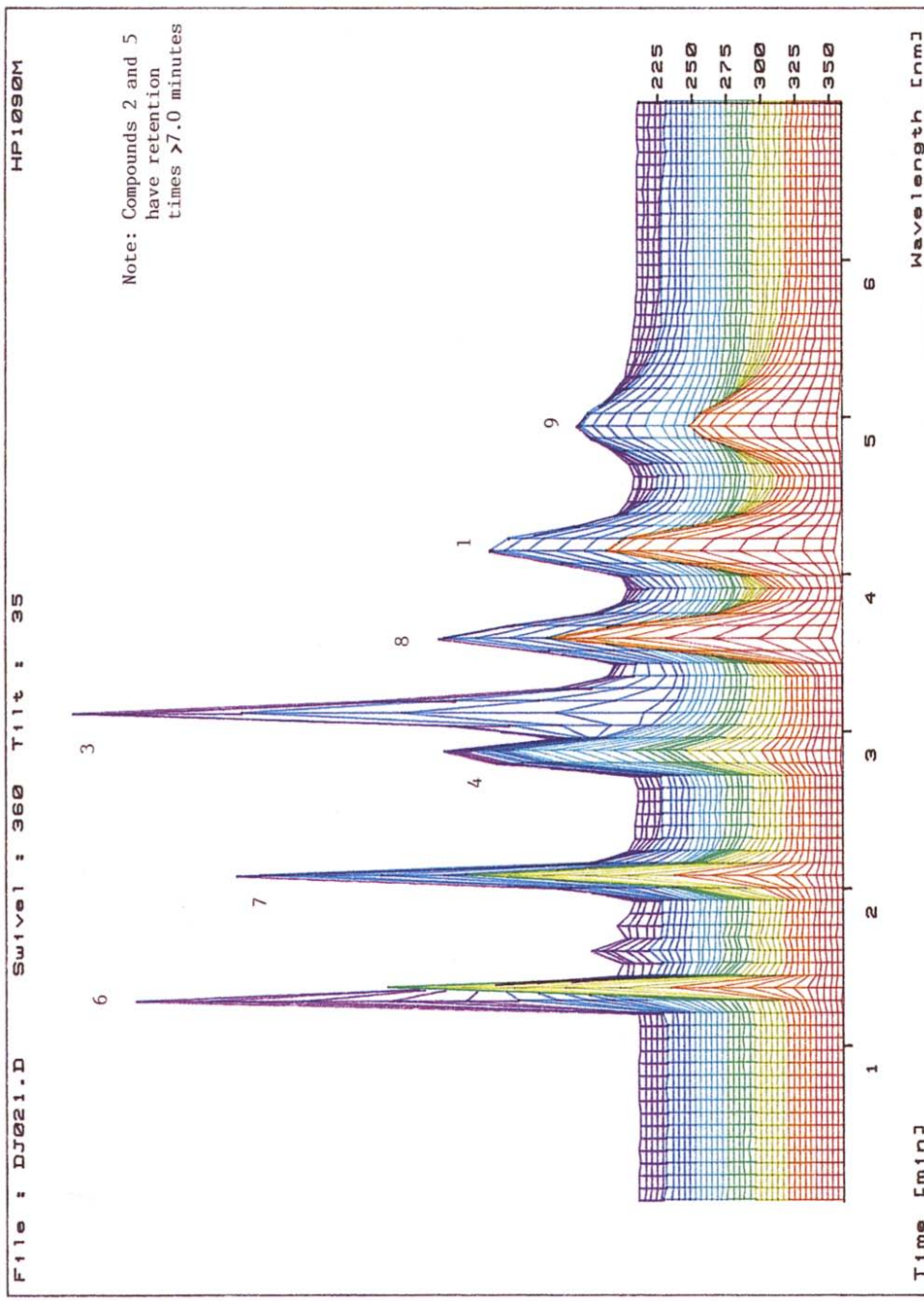

File : DJ021.D
Swivel : 360 Tilt : 35
HP1090M
Note: Compounds 2 and 5
have retention
times >7.0 minutes
6
3
7
8
1
4
9
Time [min]
1
2
3
4
5
6
Wavelength [nm]
225
250
275
300
325
350

Figure 1

Table 2
Results of assessment of the linearity of the HPLC method for the assay of ranitidine hydrochloride employing the analytical working standard dissolved in mobile phase

Experiment A — 1 µl injections			Experiment B — 10 µl injections		
Concentration of ranitidine hydrochloride (mg ml^{-1})	Concentration as % of 1 mg ml^{-1} of ranitidine	Ranitidine peak area as mean of duplicate injections (µV s × 0.25)	Concentration of ranitidine hydrochloride (mg ml^{-1})	Concentration as % of 0.1 mg ml^{-1} of ranitidine	Ranitidine peak area as mean of duplicate injections (µV s × 0.25)
0.279	25.0	457117	0.0279	25.0	516041
0.559	50.1	907915	0.0559	50.1	1033343
0.835	74.8	1368161	0.0835	74.8	1548759
1.103	99.4	1821834	0.1109	99.4	2055738
1.679	150.4	2754988	0.1679	150.4	3106981
2.234	200.2	3661236	0.2234	200.2	4139163
Correlation coefficient	0.9999		Correlation coefficient	0.9999	
Intercept (%)	−0.03		Intercept (%)	−0.41	
Equation for regression line $y = 18317.832x - 2936.000$ ($N = 6$)			Equation for regression line $y = 20675.148x - 499.2500$ ($N = 6$)		

Experiment C — 25 µl injections			Experiment D — 100 µl injections		
Concentration of ranitidine hydrochloride (mg ml^{-1})	Concentration as % of 0.04 mg ml^{-1} of ranitidine	Ranitidine peak area as mean of duplicate injections (µV s × 0.25)	Concentration of ranitidine hydrochloride (mg ml^{-1})	Concentration as % of 0.01 mg ml^{-1} of ranitidine	Ranitidine peak area as mean of duplicate injections (µV s × 0.25)
0.01116	25.0	483335	0.00279	25.0	453872
0.02236	50.1	964204	0.00559	50.1	910293
0.03340	74.8	1436765	0.00835	74.8	1358402
0.04436	99.4	1947612	0.01109	99.4	1813161
0.06716	150.4	2870382	0.01679	150.4	2736218
0.08936	200.2	3902569	0.02234	200.2	3654692
Correlation coefficient	0.9998		Correlation coefficient	0.9999	
Intercept (%)	−0.27		Intercept (%)	−0.28	
Equation for regression line $y = 19416.187x - 7150.5000$ ($N = 6$)			Equation for regression line $y = 18260.328x - 4622.2500$ ($N = 6$)		

Table 3
Typical relative HPLC retention times of ranitidine related compounds on two ODS 20 cm columns

Compound	Approximately λ_{max} in mobile phase (nm)	Relative retention times	
		Partisil ODS1	Spherisorb ODS1
Ranitidine (1)	235, 322	1.0 (4.1 min)	1.0 (3.4 min)
2	235, 322	4.9	6.1
3	235	0.8	0.6
4	217, 250	0.7	0.7
5	235	4.9	2.4
6	220, 285	0.4	0.2
7	227, 293	0.5	0.4
8	235, 322	0.9	0.6
9	235, 322	1.2	1.1

Table 4
Demonstration of the repeatability of the chromatographic stage of the HPLC assay for ranitidine hydrochloride as shown by the results of 10 replicate injections of four solutions containing the equivalent of 1 μg of ranitidine in 1, 10, 50 and 100 μl respectively

Solution A 1 μg in 1 μl		Solution B 1 μg in 10 μl	
Injection number	Ranitidine peak area (μVs × 0.25)	Injection number	Ranitidine peak area (μVs × 0.25)
1	1828738	1	2070744
2	1829433	2	2070375
3	1818809	3	2073739
4	1820086	4	2076816
5	1811635	5	2072750
6	1814815	6	2072023
7	1813028	7	2068497
8	1813297	8	2070209
9	1815750	9	2071259
10	1818871	10	2068626
Mean	1818446	Mean	2071604
RSD (%)	0.34	RSD (%)	0.22
Solution C 1 μg in 50 μl		Solution D 1 μg in 100 μl	
Injection number	Ranitidine peak area (μVs × 0.25)	Injection number	Ranitidine peak area (μVs × 0.25)
1	1936292	1	1823273
2	1939711	2	1825898
3	1937440	3	1826453
4	1939681	4	1824491
5	1943361	5	1824907
6	1941549	6	1826553
7	1938658	7	1824196
8	1937137	8	1825268
9	1937610	9	1825927
10	1940953	10	1825120
Mean	1939239	Mean	1825209
RSD (%)	0.12	RSD (%)	0.06

100 μl would apply 1 μg of ranitidine to the column, are given in Table 4. From Table 4 it is evident that precision of the chromatographic stage of the method under conditions of repeatability is good.

Corresponding experiments to test the repeatability of preparation of the solution for chromatography plus the chromatographic stages of the method, performed by injecting on to the column each of 10 solutions made up to contain 1 μg of ranitidine in 1, 10, 25 or 100 μl of the mobile phase, gave acceptably low RSD values. Values of 0.31%, 0.22%, 0.21% and 0.15% respectively were obtained, as calculated from the means of duplicate injections.

Analogous results obtained in the assessment of the precision of the HPLC method under conditions of reproducibility revealed means for the content, as calculated from the peak area ratios of the sample to the external standard, ranging from 99.0 to 99.6%, for four replicate analyses of six batches of ranitidine hydrochloride, performed on separate occasions by four different analysts using different equipment, and an overall RSD for the means of 0.7%. In analyses of 28 commercial production batches of ranitidine hydrochloride, performed at two geographical locations by different teams at different times, up to a month apart, the overall means for the content were 99.8 and 99.9% respectively, with corresponding total RSDs of 0.75% and 0.86%. From all these results, it is clear that the HPLC method of assay has an acceptable precision.

The experiments designed to test the susceptibility of the HPLC method to relatively minor changes in environmental and experimental conditions gave results indicating that it was relatively robust. Thus for continually repeated injections of two standard solutions of ranitidine hydrochloride over a 9-h run period, the RSD of the assay results was 0.3%. Similarly in analyses of six batches of ranitidine hydrochloride in duplicate on each of the column packings recommended for use in the method, the greatest difference between the means for content from the two series was 0.9%. In experiments to assess the effect of variations in mobile phase composition, molarity of the ammonium acetate solution, flow rates and batches of each of the specified stationary phases, it was found that the system suitability limits, as defined for the method, were met provided that the ratio of the methanol to the ammonium acetate solution remained within the limits 80:20 (v/v) to 95:5 (v/v) that the concentration of the ammonium acetate solution was 0.075–0.2 M, and that the flow rate was 1.5–2.5 ml per min.

The TLC method for determining related substances

Preliminary loading experiments employing commercial 20 × 20 cm plates in closed tanks presaturated with vapours of the mobile phase to eliminate "edge effects" and irregular development of the chromatograms [49, 50], revealed that a loading of 223 μg of ranitidine hydrochloride AWS (equivalent to 200 μg of ranitidine), resulted in neither distortion of spot shape nor streaking. At this loading, related impurities at 0.2 μg would represent 0.1% of the nominal sample loading.

Two-dimensional TLC was applied to a sample of ranitidine hydrochloride stored at 30°C and 50% RH for 30 months. Chromatography in the first direction employed the mobile phase defined for the method. Chromatography in the second direction employed a mobile phase of markedly different polarity, namely a mobile phase comprising chloroform, ethanol, ammonia and water. Upon visualisation of the chromatogram, it was seen that all spots entering the chromatography in the second direction remained as single spots after completion of the chromatography. This permits the conclusion that

none of the products of degradation of ranitidine hydrochloride were co-migrating in the defined chromatographic system.

It was then shown that, within the time limit imposed upon the application of the sample solutions for the method, there was no degradation on the chromatoplate of any of the compounds of Table 1.

Results from challenge experiments to demonstrate the complete separation of all the compounds listed in Table 1 on the defined TLC system, together with their limits of detection, relative response factors and relative retardation factors are shown in Table 5. In particular successive loadings of decreasing amounts of ranitidine hydrochloride revealed that the threshold of detection of the ranitidine liberated by the ammonia in the mobile phase occurred at about 0.025% of the defined loading of 223 μg of ranitidine hydrochloride.

Table 5
Thin-layer chromatographic parameters for the compounds of Table 1

Compound	Relative R_f (Ranitidine = 1.00)	Relative response factor (Ranitidine = 1.00)	Limit of detection (as percentage of the standard loading of 223 μg of ranitidine hydrochloride)
1	1.00	1.00	0.025%
2	1.3	1.0	0.1%
3	1.4	0.6	0.1%
4	0.9	1.0	0.1%
5	1.1	1.3	0.1%
6	0.2	0.4	0.1%
7	0.4	0.8	0.05%
8	0.2	0.8	0.05%
9	0.5	0.8	0.05%

This led to the incorporation, within the three system suitability checks built into the method, of the use of two standard applications (one adjacent to each vertical side of the chromatoplate) of ranitidine hydrochloride, analytical reference standard, at a loading of 0.05% of the nominal sample loading. The chromatography is shown to be valid when each of these two sensitivity checks in the developed chromatogram is visible and when both show equal intensity. The remaining two system suitability checks were: (a) the demonstrable complete separation of the spot, due to compound 5 in Table 1 from the spot, due to ranitidine in the developed chromatogram upon co-chromatography of the two (resolution check); and (b) confirmation that the major drug related impurity present (at <0.5%) was compound 2 in Table 1 (impurity identity check), thus confirming conformity of the quality of the sample with that of the material used in the safety studies.

The reproducibility of the TLC method was shown by having three analysts independently determine, on different days, the related substances present in a batch of ranitidine hydrochloride which had been stored at 30°C and 50% RH for 30 months. Two analysts found the principal impurity to be present at 0.5% m/m of the standard loading of 223 μg of ranitidine hydrochloride, whereas the third analyst found 0.4% m/m. All three analysts found the second most abundant impurity to be present at a level of 0.3% m/m.

Conclusions

From this work, it is possible to define robust, accurate, precise and sensitive chromatographic methods, which when used in conjunction, will provide reliable assessment of the identity, strength and purity of ranitidine hydrochloride — both in the drug substance and in its dosage forms.

Definition of the HPLC assay procedure

The procedure includes confirmation of the suitability of a stainless steel tube (200 or 250 × 4.6 mm i.d.) packed with a stationary phase of ODS bonded to silica particles of diameter 10 μm (both Sperisorb ODS and Partisil ODS of the type optionally designated "1" have been found suitable), for the assay of ranitidine hydrochloride by test chromatography. Using a mobile phase consisting of a mixture of methanol and 0.1 M ammonium acetate, in a ratio of 80:20 (v/v)–95:5 (v/v) (typically 85:15, v/v) inject, via an appropriate fixed volume loop, 1.12 ± 0.05 μg of ranitidine hydrochloride, AWS, in admixture with 0.02 μg of *N*-[2-[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]sulphinyl]ethyl]-*N'*-methyl-2-nitro-1,1-ethenediamine, dissolved in 1–50 μl of mobile phase. This solution is not to be used after 3 months following its preparation. Perform the chromatography at 15–30°C at a flow rate of 1.5 to 2.5 ml min^{-1}, measuring the responses by means of a u.v. detector set at 322 nm. The column is suitable for use for the assay of samples of ranitidine hydrochloride by external standardisation using the ranitidine hydrochloride AWS, employing the same mobile phase composition, sample concentration, injection volume, temperature and flow rate, as were used in the test chromatography where the results from this test chromatography reveal, with respect to the peak arising from the ranitidine hydrochloride AWS, that: (a) the resolution, R_s, from the peak due to ranitidine-S-oxide is not less than 1.5; (b) the number of theoretical plates, N, is not less than 3500 per m; (c) the tailing factor, f, is not greater than 2.0; (d) the retention time, t_r, is 2.8–5.0 min; and (e) the RSD over eight replicate determinations is less than 2.0%.

Definitions employed are:

$$R_s = \frac{1.18\,(t_{r2} - t_{r1})}{W_1 + W_2} \qquad N = 5.54\left(\frac{t_r}{W}\right)^2 \qquad f = \frac{b}{a},$$

where W is the peak width at half the peak height; b is the back half-width at 10% of the peak height; a is the front half-width at 10% of the peak height, t_r is the retention time and where subscripts 1 and 2 are used to denote the first and second peaks of interest, respectively.

A column meeting these criteria for the assay of samples of ranitidine hydrochloride drug substance and its dosage forms is employed. Solutions for the assay are to be prepared by dissolving the sample of drug substance in mobile phase, through preparing solutions in mobile phase of the drug extracted from solid dosage forms, and through dilutions with mobile phase of liquid dosage forms, so as to secure concentrations within these sample solutions in terms of the nominal content of the drug (100% for the drug substance and label claim for the dosage forms), which will give a nominal loading on the column of about 1 μg of ranitidine when injection is made of from 1 to 50 μl of the solution on to the column by fixed volume injection loop.

Standard solutions of ranitidine hydrochloride AWS in the mobile phase are

accurately prepared to a precision of ±0.5%, and to the same concentration as the nominal value for the sample solutions using appropriate volumetric glassware.

The assays of the sample solution are conducted by applying the fixed volume to the column interspersed with injections of the standard solutions, to a statistical pattern so that no more than six sample solutions are applied in uninterrupted sequence, before there is application of a standard. All peak areas are measured. The response factor, R, for each standard is calculated from the equation:

$$R = \frac{Ws}{As} \times \frac{P}{100},$$

where Ws is the weight (mg) of ranitidine hydrochloride AWS used to make up the standard solution; As is the area of the peak obtained with the injected volume from that solution, and P is the percentage purity of the AWS (m/m). A mean response factor, $\bar{R}$, is calculated for all the standards injected. The ranitidine hydrochloride content of each sample solution, q, is then calculated from the equation:

$$q = \frac{100\bar{R} \times Au \times DFu}{Wu \times DFs} \%\text{m/m},$$

where Au is the area of the peak obtained with a given sample injection (or the average area of the two peaks where there are duplicate sample injections), Wu is the weight (mg) of the sample of ranitidine hydrochloride used to make the sample solution and DFs and DFu are the dilution factors of the standard and sample solutions, respectively. For the determination to be acceptable, the area of the larger of the two peaks obtained with duplicate sample injections has to be not more than 1% greater than that of the smaller peak.

Definition of the TLC procedure for determining related substances

Chromatography is to be conducted in a tightly sealed glass chromatotank, lined with chromatographic paper and pre-equilibrated for at least 1 h with the vapours, from about 200 ml of the mobile phase which comprises ethyl acetate–isopropanol–ammonia solution (wt per ml = 0.880 g; 35% m/m)–water (25:15:4:2, v/v/v/v). The mobile phase is used within 6 h of preparation and is to be freshly prepared for each chromatogram. Chromatoplates used are silica gel $60F_{254}$, 0.25 mm thick, typically 20 × 20 cm.

Chromatograms are run at ambient temperature. To ensure adequate performance of the chromatography, application of a resolution check solution (not more than 3 months old) of 5-[[(2-aminoethyl)thio]methyl] *N*,*N*-dimethyl-2-furanmethanamine hemifumarate (0.127% m/v in methanol), together with two applications of standards of ranitidine hydrochloride at 0.05% of the nominal loading (223 μg) of the sample under investigation are made to the chromatoplate. Additionally, an impurity identity check solution is applied, consisting of a 0.1% m/v solution (not more than 3 months old) of *N*,*N*′-bis[2-[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]-2-nitro-1,1-ethenediamine in methanol, to ensure that the major related substance present is that for which full safety data has been generated. The level of this compound is restricted to not more than 0.5% of the sample loading.

Applications of the nominal 223 μg loadings of the samples are to be performed as rapidly as possible, preferably to a left-to-right lateral pattern, comprising a block of five

lower loadings of the standards; resolution check; block of up to six samples; identity check; block of the three higher loadings of the standards; and repeat loading of the lowest standard. Development of the chromatogram should not be started before all solvents have evaporated from the spot applications, but should be performed within 20 min of the first sample application. Loadings of standards corresponding to 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 1.0 and 0.05% of a true sample loading of 200 μg of ranitidine (223 μg of ranitidine hydrochloride), which is the nominal loading of sample applied to the plate, are made in the same volumes as that used to apply the samples. Applications of the two loadings of 0.05% are made adjacent to the opposite vertical sides of the chromatoplate. The diamine resolution check solution is applied as a 2-μl aliquot, on top of which is applied 10 μl of an additional sample solution. The identity check solution is applied as a 10-μl aliquot as are all sample solutions. The chromatogram is developed until the solvent front has travelled 15 cm. Detection, after removal of the chromatoplate from the development tank and allowing it to dry in a current of air at room temperature, is by exposure to iodine vapour for about 15 min in a sealed, dry TLC tank, previously equilibrated at 30°C for at least 1 h after insertion of 5 g of iodine crystals in two open petri dishes.

The chromatography can be considered valid only if: the standards of ranitidine hydrochloride at the loading of 0.1 μg at either end of the plate have been visualised and have equal intensity; and where there is full separation of the spots due to "diamine" (5) and ranitidine within the track resulting from application of the resolution check.

Quantitation of the related substances is conducted immediately after removal of the chromatoplate from the iodine vapour, through visual comparison of the intensity of any spots, other than that due to ranitidine, present in the sample tracks with the spots in the standard tracks, employing a two-point bracketing approach. The white spot occurring close to the baseline in the sample tracks is ignored, as it has been shown to be due to ammonium chloride liberated from the ranitidine hydrochloride by reaction with the ammonia present in the mobile phase.

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