ELSEVIER

Journal of Chromatography B, 701 (1997) 87–95

JOURNAL OF CHROMATOGRAPHY B

Development of an assay for the simultaneous determination of sildenafil (Viagra) and its metabolite (UK-103,320) using automated sequential trace enrichment of dialysates and high-performance liquid chromatography

J.D.H. Cooper[a,*], D.C. Muirhead[b], J.E. Taylor[a], P.R. Baker[a]

[a]*Clinical Innovations Ltd., Building 28, Stoneleigh Deer Park, Stareton, Kenilworth, Warwickshire CV8 2LY, UK*
[b]*Early Clinical Research Group, Pfizer Central Research, Pfizer Ltd., Sandwich, Kent CT13 9NJ, UK*

Received 23 December 1996; received in revised form 3 June 1997; accepted 18 June 1997

Abstract

The development of the ASTED (automated sequential trace enrichment of dialysates) system to prepare plasma samples prior to high-performance liquid chromatography (HPLC) of sildenafil and its demethylated metabolite (UK-103,320) is described. Investigations to elucidate potential pitfalls of the ASTED on-line sample preparation system prior to separation of the analytes by HPLC are presented. The procedure is shown to be selective for sildenafil and UK-103,320, and linear over the range 1.00–250 ng/ml. The intra-batch imprecision (C.V.) of the method at plasma analyte concentrations of 1.00, 5.00, 50.0 and 200 ng/ml was 11.2, 3.10, 1.50, and 1.30%, respectively, and the corresponding inter-batch imprecision was estimated to be 13.5, 7.09, 3.69, and 5.43%. At these plasma analyte concentrations the overall inaccuracy (% bias) of the procedure ranged from 3.6 to 8.4%. The method showed similar assay performance for the estimation of the metabolite, UK-103,320. The application of the assay to a pharmacokinetic investigation during a clinical study is presented. © 1997 Elsevier Science B.V.

Keywords: Trace enrichment; Sildenafil; UK-103,320

1. Introduction

A reliable and specific assay is important for characterisation of a drug's disposition, tolerance and safety. Sildenafil((1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo- 3 -propyl-1H-pyrazolo-[4, 3-d]pyrimidin-5-yl)phenylsulphonyl]-4-methylpiperazine), currently undergoing pharmacokinetic, efficacy and safety evaluation by Pfizer Central Research [1], is a potent and selective inhibitor of cGMP (type V)-specific phosphodiesterase capable of enhancing the relaxation of the penile corpus cavernosum and therefore having the potential to improve penile erectile function. Its metabolite, UK-103,320 (1-[4-ethoxy-3-(6, 7-dihydro- 1 -methyl- 7 -oxo- 3 -propyl-1H-pyrazolo [4, 3-d]pyrimidin - 5 -yl)phenylsulphonyl-piperazine) is present at about 30–40% of the plasma parent drug concentrations observed in subjects after single oral dosing. UK-103,320 appears to have a similar potency to sildenafil and hence may contrib-

*Corresponding author.

0378-4347/97/$17.00
PII S0378-4347(97)00339-3

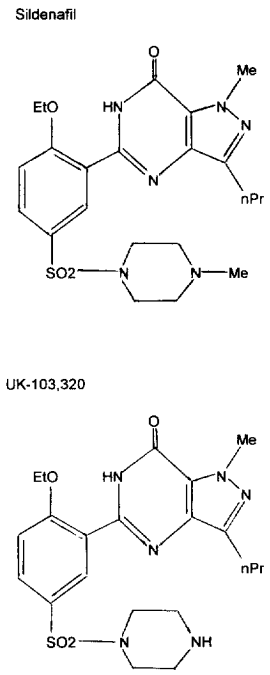

Fig. 1. Structures of sildenafil and its metabolite UK-103,320.

ute to pharmacological effects (S.A. Bollard, Pfizer Ltd., UK, unpublished data). Quantification of both compounds is therefore essential during the evaluation of the parent drug.

Both sildenafil and UK-103,320 (Fig. 1) have basic functional groups with a pK_a value of 8.7, although a weak acidic moiety is also present on the parent compound. Difficulties may arise during the analysis of compounds with basic properties. Adsorption of such compounds by exposed silanols on silica HPLC column materials is well known [2], and problems could be possible during the collection and preparation of biological samples such as plasma. Non-specific binding of ionic species to storage containers has been recognised [3] and losses may occur, especially during transfer of extracts for various sample preparation procedures.

Although numerous reports [4–7] concerning the application of ASTED to a wide variety of compound classes have appeared, few have addressed the difficulties of on-line systems with respect to the analysis of organic bases. ASTED configurations with increased sample preparation capabilities have been described in earlier publications [8,9]. This paper reports the development and evaluation of the ASTED system for the simultaneous estimation of plasma sildenafil and its metabolite, UK-103,320. A further compound, UK-108,302, was also included in the application to aid assessment of sampling efficiency during the automated ASTED process, but was not incorporated for quantification purposes. Results obtained during validation of the procedure and its application to a pharmacokinetic study are presented.

2. Experimental

2.1. Instrumentation

The HPLC and ASTED units were obtained from Anachem (Luton, UK).

2.1.1. HPLC

The isocratic HPLC system consisted of a Gilson Medical Electronics (Villiers-le-Bel, France) 306/5SC pump, a 118 UV detector and a Rheodyne (Cotati, CA, USA) 7010 injection valve fitted on the ASTED unit. Control of the HPLC system, integration of chromatographic peaks and communication with the ASTED system (via Gilson GSIOC) was achieved using a 715, V1.2, system controller and Microsoft Windows software V3.11 located in a 486 PC.

2.1.2. Sample preparation (ASTED) unit

The software and hardware modifications to an ASTED unit to enhance its capabilities have been described previously [8]. The modified ASTED unit (Fig. 2) comprised a 231 auto-sampling injector; two 401 dilutors fitted with 1-ml syringes (controlling sample pre-treatment on the donor side and dialysate flow on the recipient side of the dialyser); a universal valve switching module (UVSM) to isolate the dialyser unit from the trace enrichment device; a three-way 12 V d.c. valve to enable two different reagents to be delivered by a 401 dilutor controlling sample delivery to the system and two serially connected Kel F dialyser units with an approximate 750-μl total donor volume fitted with 15 kDa Cuprophan membranes (regenerated cellulose from Enka, Germany), and a stainless-steel trace enrich-

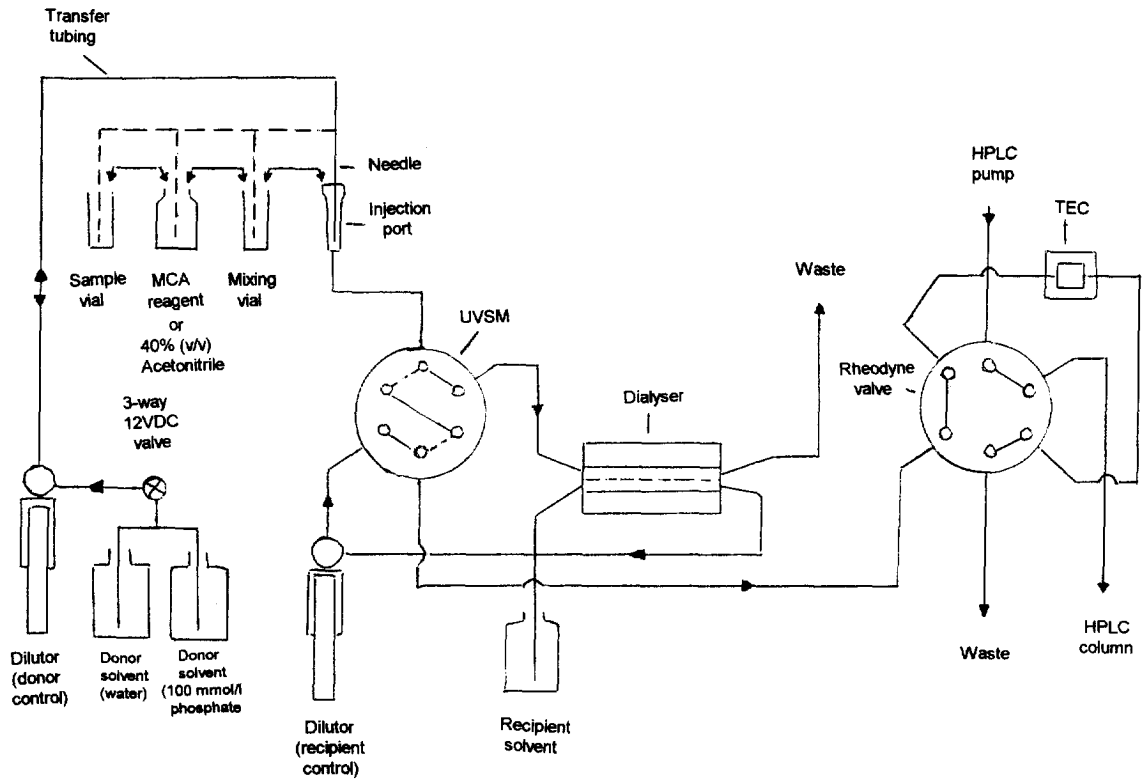

Fig. 2. Schematic diagram of the ASTED system used for the analysis of sildenafil and its metabolite UK-103,320. The arrows indicate flow direction of reagents.

ment cartridge (TEC) packed, in-house, with 70 mg of 10 μm Hypersil C1 (Shandon Southern Products, Runcorn, UK) to replace the loop on the Rheodyne 7010 injection valve. Control of the ASTED operations used specialised software (V1.10, Clinical Innovations, Kenilworth, UK). The internal surfaces of the transfer and connection plastic tubings used to move liquids on the ASTED system were chemically pre-treated [8] to increase the velocity of liquid flow without disrupting sample integrity.

2.2. Optimised reagent conditions

2.2.1. General reagents

All chemicals were analytical grade obtained from Sigma (Poole, UK). HPLC-grade water, prepared using a Purite (Thame, UK) system, was used for all reagent preparations. HPLC solvents (high purity) were obtained from Romil (Cambridge, UK). The following reagents were prepared: potassium hydrogen orthophosphate buffer (500 mmol/l, pH 4.5 and 7.0); 40% (v/v) acetonitrile in water; 1.0 mol/l monochloroacetic acid (MCA) containing 200 ng/ml UK-108,302 as a reference compound in 10% (v/v) methanol. Potassium phosphate buffer (100 mmol/l, pH 7.0) or water were dispensed by the Gilson 401 dilutor on the donor channel of the dialyser. Potassium phosphate buffer (10 mmol/l, pH 7.0) containing 10% (v/v) methanol was dispensed by the second Gilson 401 dilutor into the recipient channel of the dialyser.

2.2.2. Preparation of calibration standards

Individual 100 μg/ml stock solutions of sildenafil (citrate salt) and UK-103,320 (base) were prepared in methanol. From these, working standards containing 0.10 and 2.50 μg/ml of sildenafil and UK-103,320 were prepared in 20% (v/v) methanol–

water. Six calibration standard concentrations ranging from 1 to 250 ng/ml of both analytes, calculated as free base, were prepared by supplementing blank plasma with the working standards. The final methanol concentration in plasma was less than 0.2% (v/v).

2.3. Optimised chromatographic conditions

An isocratic HPLC mobile phase was utilised, comprising acetonitrile–potassium phosphate buffer (500 mmol/l, pH 4.5)–water (28:4:68, v/v/v) at a flow-rate of 1.5 ml/min. Diethylamine hydrochloride (DEA), 10 mmol/l, was dissolved in the buffer/water prior to the addition of acetonitrile. The mobile phase was de-gassed with helium before use. The HPLC column (100×4.6 mm I.D.) was packed with 5 μm Kromasil C_4 (Technicol, Stockport, UK). No guard column was employed and the analytical column was maintained at 40°C in a column block heater. The detector was set at 230 nm wavelength, absorbance maximum for sildenafil, and an absorbance range of 0.001 AUFS.

2.4. Optimised ASTED sample preparation conditions

Sample preparation was carried out in a completely automated manner using the ASTED version A system as follows: (1) 650 μl of plasma were mixed with 150 μl of MCA-reference compound and 780 μl of this mixture delivered into the donor channel of the dialyser; (2) recipient solvent (7000 μl) was moved through the TEC in a 6-min time period; (3) following enrichment the donor tubing and dialyser channel were purged with 1500 μl of donor solvent (water) and 200 μl of 40% acetonitrile, followed by 1000 μl of donor solvent moved (via UVSM valve switching) through the TEC; (4) the Rheodyne high pressure valve was switched to the inject position and the analyte eluted onto the HPLC column by the HPLC mobile phase; (5) the three-way 12 V d.c. valve on the inlet of the 401 dilutor was switched to enable the system to be purged with 9 ml of 100 mmol/l phosphate buffer (pH 7.0) through the donor channel and, simultaneously, 9 ml of methanol–buffer through the recipient channel via the second 401 dilutor. At the completion of this cycle the three-way 12 V d.c. valve on the inlet of the 401 (donor) dilutor was switched to enable the system to be further purged with 4 ml of water through the donor channel and, simultaneously, 4 ml of methanol–buffer through the recipient channel via the second 401 dilutor; (6) the TEC was then regenerated with 500 μl of recipient solvent ready for the next sample.

2.5. Quantification

Calibration standards were sited at the beginning and end of each analytical batch. A linear regression (weighting $1/X^2$) was performed on the peak areas and concentrations of each analyte. The regression lines established were used to calculate test analyte concentrations by interpolation. The automatic addition of the reference compound (UK-108,302) was included as an indicator that correct sampling had occurred and not for quantification purposes.

2.6. Control samples

Drug-free plasma, supplemented with 1.00, 5.00, 50.0 and 200 ng/ml of sildenafil and UK-103,320 was aliquoted and stored at −20°C. Six different plasma samples were also supplemented with 50.0 ng/ml of both compounds. The sildenafil and UK-103,320 methanol–water solutions used to supplement the plasma were prepared from separate weighings to those solutions used to prepare the calibration standards. As for the calibration standards, the final methanol concentration in plasma was less than 0.2% (v/v).

3. Results

3.1. Optimisation of chromatography and sample preparation conditions

3.1.1. Chromatography conditions

An elution pH of 4.5 was required for separation of sildenafil and UK-103,320 and the reference compound, UK-108,302 (Fig. 3). The inclusion in the elution solvent of a silanol blocking amine (DEA) together with the use of a high carbon loaded

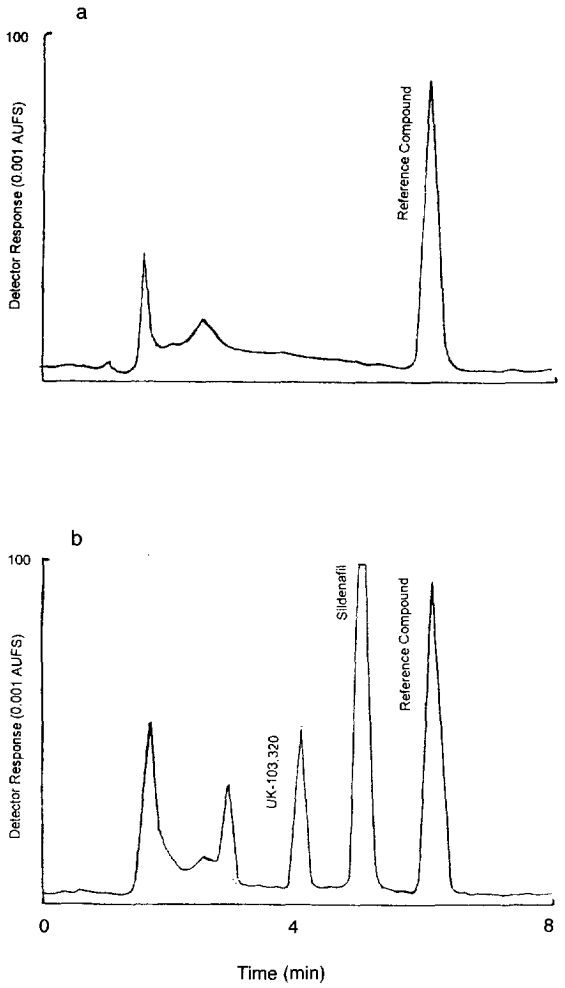

Fig. 3. Chromatograms of (a) prepared blank plasma and (b) prepared 2-h post-dose plasma from a subject receiving 50 mg of sildenafil as a single oral dose. The measured plasma sildenafil and UK-103,320 concentrations were 71.3 and 19.6 ng/ml, respectively.

HPLC column ensured symmetrical peak shapes of the compounds under investigation.

3.1.2. Sample preparation conditions

Two dialyser units connected in series and correct selection of trace enrichment times were required to obtain sufficient sensitivity for accurate and precise estimation of the compounds at the lower limit of quantification (i.e. 1.00 ng/ml). To avoid sample-to-sample interactions, the fluid transfer tubings on the ASTED system were purged with phosphate buffer (pH 7.0, 100 mmol/l). Switching to a water purge was then essential to avoid breakthrough of the compounds from the trace enrichment device during an acetonitrile–water wash cycle. This was essential to achieve assay specificity. The use of concurrent sequential analysis, i.e. the automated preparation of a sample during the chromatography of the previously prepared specimen ensured good sample throughput.

3.2. Validation of assay performance

3.2.1. Linearity and limits of quantification

Peak areas varied linearly over the analytical range employed (1.00–250 ng/ml per each compound). The lower quantification limit was set at the lowest standard concentration on the calibration curve.

3.2.2. Imprecision and bias

The inter-batch imprecision and overall inaccuracy (%bias) were determined by replicate analysis ($n=6$) of each control plasma analyte concentration over four separate analytical batches. The results are shown in Table 1 together with the intra-batch imprecision data from one of the analytical batches.

3.2.3. Assay specificity

Drug-free lipaemic plasma and drug-free plasma samples from six volunteers were assayed using the procedure described. No chromatographic interferences were observed as typified by a chromatogram from the preparation of a blank plasma (Fig. 3a). Solutions of other known metabolites of sildenafil were also analysed together with prepared solutions of salicylate, propranolol, nifedipine and captopril, and no chromatographic interferences with the assay were observed.

3.2.4. Sample matrix effects

The accuracy (C.V.%) of the method determined by analysing blank plasma samples from volunteers each supplemented with 50.0 ng/ml of both sildenafil and UK-103,320 was estimated to be 113 (2.8) and 106 (1.80) for sildenafil and UK-103,320, respectively, indicating that sample matrix effects do not affect inter-assay imprecision. This was also measured using standards prepared in aqueous/methanol solution giving an overall accuracy of 103

Table 1
Intra- and inter-assay imprecision and inaccuracy of the ASTED–HPLC method for the analysis of sildenafil and UK-103,320 in plasma

Analyte	Spiked plasma conc. (ng/ml)	Intra-assay imprecision and inaccuracy			Inter-assay imprecision and inaccuracy		
		Estimated mean conc. (ng/ml)	C.V. (%)	Bias (%)	Mean	C.V. (%)	Bias (%)
Sildenafil	1.00	1.06	11.2	6.0	1.11	13.5	11.0
	5.00	5.37	3.10	7.4	5.18	7.09	3.6
	50.0	55.1	1.50	10.2	53.3	3.69	6.7
	200	226	1.30	13.0	220	5.43	8.4
UK-103,320	1.00	1.03	8.20	3.0	1.09	13.6	9.0
	5.00	5.05	4.90	1.0	4.82	7.16	−3.6
	50.0	48.9	1.80	−2.2	47.9	3.49	−4.3
	200	208	1.10	4.0	202	4.80	1.5

and 104% for sildenafil and UK-103,320, respectively.

3.2.5. *Instability of the analytes*

Using the ASTED–HPLC assay procedure no obvious degradation of the compounds in plasma over 24 h at room temperature was observed. Similar investigations were conducted for aqueous solutions of the analytes stored in standard polypropylene ASTED vials and in glass. Fifty percent losses of both compounds were observed within a 30-min time period after storage in plastic compared with a 2% loss in glass. The losses in plastic were negated by including 20% (v/v) methanol in the aqueous solution.

3.2.6. *Sample carry-over*

Carry-over was determined by analysing blank plasma following the assay of a calibration standard containing 250 ng/ml of each analyte. This was performed in triplicate and the carry-over estimated to be <0.3% in each case.

3.2.7. *Pharmacokinetic investigations*

Biological samples from numerous phase I and II clinical studies have been assayed using the ASTED–HPLC procedure. As an example, the application of the method to the analysis of plasma samples from a subject receiving 50 mg of sildenafil as a single oral dose, during an open, randomised, three-way crossover study is presented. Blood samples were collected pre-dose and at intervals up to 24 h post-dose. Plasma was stored at −20°C until analysis. A chromatogram from this study is shown in Fig. 3b. A further peak (UK-150,564) has been identified as a further metabolite of sildenafil. This can be seen in Fig. 3b with a retention time of approximately 3.0 min and was not quantified. The pharmacokinetic profile from the analyses is shown in Fig. 4.

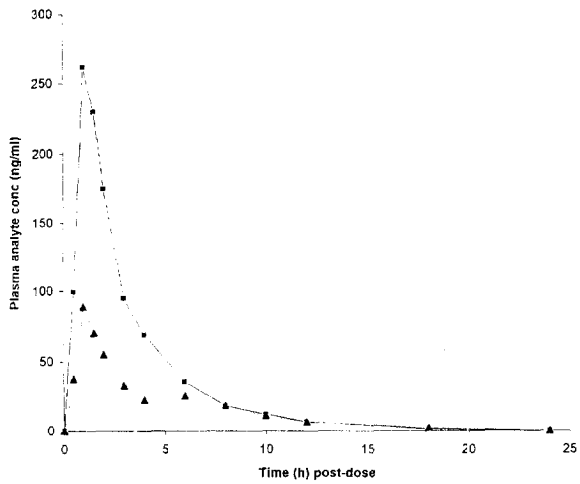

Fig. 4. Pharmacokinetic plot of plasma sildenafil (■) and UK-103,320 (▲) concentrations from a subject receiving 50 mg of sildenafil as a single oral dose during an open, randomised, three-way crossover study.

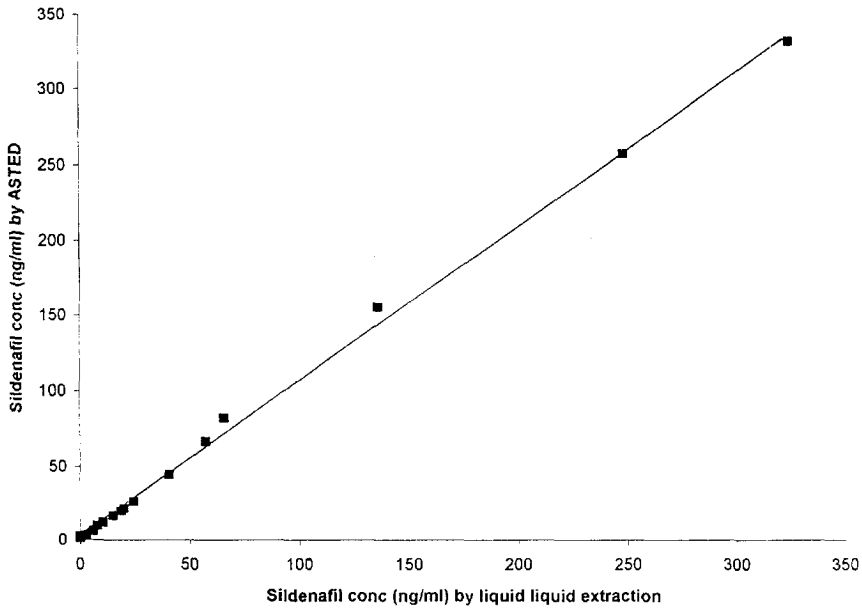

Fig. 5. Comparison of ASTED and a liquid–liquid extraction method for the estimation of sildenafil in plasma. For sildenafil the regression line parameters were calculated as $Y=1.03X+2.88$ ($r=0.9982$). Similar results for the analysis of UK-103,320 were observed with regression parameters calculated as $Y=1.08X+1.25$ ($r=0.9977$).

3.2.8. Comparison of ASTED–HPLC with liquid–liquid extraction method

Twenty plasma samples obtained from healthy subjects and patients with mild-to-severe renal failure, receiving single doses of sildenafil, were analysed by the ASTED–HPLC and a benzene extraction procedure (Nagoya, Pfizer Inc., Japan, unpublished assay). Fig. 5 shows a comparative plot of the data for sildenafil. The results for UK-103,320 using these two sample preparation approaches showed similar scatter.

4. Discussion

Discussions concerning the importance of sample preparation and the attributes of on-line systems such as ASTED have been previously published [4]. The analysis of compounds having basic functional groups in biological samples using automated systems may be problematical due to the non-specific binding characteristics of organic bases to plastic surfaces within the assay system either by ionic or hydrophobic interactions.

4.1. ASTED sample preparation

4.1.1. Non-specific binding of bases during sample preparation

Automating procedures using relatively inexpensive Cartesian *xyz* robots suffers from the major disadvantage that all sample/liquid movements pass through single line transfer tubing. However, all robotic systems can suffer problems when analysing organic bases as demonstrated by the loss of sildenafil and UK-103,320 in aqueous solutions stored in polypropylene vials during these investigations. That this can be attributed to non-specific binding of the compounds to plastic surfaces can be surmised by the low losses observed with storage in glass. These difficulties are generally overcome when the analytes are retained in plasma, especially when high protein binding occurs as is the case for sildenafil and UK-103,320 (unpublished data). However the

problems of solution storage in vials can be amplified in the case of automation within systems using single line transfer tubing. Protein build-up on plastic surfaces and the binding of bases to both protein and exposed plastic binding sites can cause significant sample-to-sample interactions and, as a result, unreliable assays arise due to carry-over. Carry-over can become a rate-limiting factor in that very large purge volumes are required to eliminate the interference by the previous sample. In this assay carry-over was minimised by purging with a high concentration of phosphate buffer (pH 7.0, 100 mmol/l).

4.1.2. Optimisation of the ASTED parameters

4.1.2.1. *Dialysis parameters.* It is well known that dialysis is a relatively slow process which has been accelerated by the ASTED approach using continual movement of the recipient solvent. This improves sample throughput and detection limits [4]. Although the rates of transfer are increased dilute analyte concentrations in the dialysate ensue and enrichment is usually required. Combining the two processes, absolute recoveries of greater than 50% using smaller dialysis units with donor volumes of 100 μl have been achieved within short time periods [4]. For sildenafil and UK-103,320, low limits of 1.00 ng/ml in plasma were desirable and the low molar absorptivity of these compounds dictated that small dialyser units could not be utilised. In this assay two large dialyser units connected in series were required with a total donor capacity of 750 μl. Although this enabled reasonable sample throughput times (four per hour) a relative reduction in recovery (measured at 30%) of the analytes was observed compared with quoted values of 50% [5] using smaller-pathlength dialyser units. This was due to reduced volumes of recipient solvent being exposed to the membrane surface, reducing the rate of transfer of the compounds. However the target quantification limits (1.00 ng/ml) could still be achieved due to the efficiency of the sample preparation process producing chromatographic separations with few background interferences (Fig. 3a).

4.1.2.2. *Trace enrichment parameters.* Unlike the problems associated with non-specific binding of bases, the ion-exchange properties of these compounds can be used to advantage. The use of non-end-capped Hypersil short-alkyl chain silica ensures that enrichment is probably due to ion-exchange mechanisms between the positively charged amine moiety of the bases and the exposed negatively charged silanols. With such mechanisms good assay specificity is achieved by washing the enriched compounds with organic solvents (e.g. acetonitrile) to remove non-polar interferences. For the model used in this assay, 200 μl of 40% (v/v) aqueous acetonitrile gave sufficient clean-up without reducing the breakthrough volume of sildenafil to such levels that losses occurred. Breakthrough of sildenafil was observed with acetonitrile volumes in excess of 500 μl. For UK-103,320 and other stronger bases, increased acetonitrile strengths (up to 60%) can be used with the inclusion of buffers without rendering a loss in breakthrough volume. Such effects suggest that the clean-up can be governed by mixed mechanisms of ionic and hydrophobic interactions. Efficient clean-up can also be dependent on the pH and nature of the recipient solvent containing the dialysate. Like the low analyte concentrations in the accelerated dialysis procedure, the MCA concentrations are much reduced in the dialysate compared with the original concentrations of approximately 160 mmol/l in the donor channel of the dialyser. A 10-mmol/l phosphate solution (pH 7.0) was sufficient in this procedure to buffer reduced MCA concentration in the dialysate. Enrichment of the dialysate at pH 7.0 avoided chromatographic interferences.

4.1.2.3. *Sample preparation and matrix effects.* Between-sample matrix variations can have deleterious effects on assay performance for many biological assay procedures. This is particularly the case for highly protein-bound compounds. For measurement of total drug concentrations, elimination of protein binding is the ideal especially when membranes are involved in the sample preparation and a number of alternatives are available. Altering the pH of plasma has been shown to be beneficial for molecules such as xanthines [4], and this proved the case for sildenafil and UK-103,320. The selection of MCA to achieve this objective was made since its addition to plasma reduced the pH (approximately 3.5) suffi-

ciently without visually precipitating proteins, a prerequisite for movement of liquids through any on-line system analysing biological fluids. The relative recovery of the analytes from aqueous solution compared with plasma treated with MCA suggests that the assay is not compromised by protein binding, and that between-sample variations are not problematical. In view of the addition of methanol to the plasma with the MCA reagent and the back dialysis of methanol from the recipient it can be assumed that the low concentration of methanol added with sildenafil and UK-103,320 to plasma to prepare standard and control samples is negligible. It can therefore be postulated that volunteer plasma samples behave in a similar manner. To some extent this is confirmed by comparison of the assay with a liquid–liquid extraction procedure (see Section 4.3) and the recovery data obtained when supplementing six different plasma samples with 50.0 ng/ml sildenafil and UK-103,320. The slight positive bias shown with these recoveries is mirrored by the bias shown in the imprecision investigations (Table 1) and is probably due to differences in the make-up of the control samples compared with the calibration standards. The direct analysis of plasma without the addition of MCA could perceivably permit the direct estimation of free (non-protein-bound) estimations of the compounds and work is now underway to confirm this method potential.

4.2. Application to pharmacokinetic investigations

The method described has been successfully applied to the measurement of sildenafil and metabolite plasma concentrations in samples from many pharmacokinetic studies involving young healthy subjects, patient groups suffering with pathological impairments (diabetic, hepatic and renal) and elderly healthy subjects receiving oral sildenafil. An example of a pharmacokinetic profile obtained using the ASTED assay procedure is shown in Fig. 4. There have been virtually no false-positives for pre-dose samples and no known cases of analytical interference by endogenous or exogenous substances. Throughout the bioanalytical programme the method has performed well with respect to accuracy and imprecision as assessed from quality control and calibration data.

4.3. Assay performance

The ASTED procedure has been shown to be highly specific (Fig. 3a) for the compounds under investigation using HPLC as the means to separate and quantify the analytes. Using the ASTED and HPLC assay conditions described, Gaussian peak shapes were observed (Fig. 3b) and several thousand injections of prepared plasma samples were achieved without extensive loss of column efficiencies. Combined with this, several hundred injections of prepared plasma samples can be made using the same trace-enrichment device ensuring robust and economical procedures. The acceptable assay imprecision (Table 1) without internal standardisation is achievable due to the on-line nature of the process. Such procedures offer advantages of simpler chromatographic separations. The data obtained (Table 1) indicate that the procedure was accurate and this was substantiated by comparison with a liquid–liquid extraction technique (Fig. 5). The use of this technique has provided rapid means for the analysis of sildenafil and UK-103,320 and the acceptable assay performance has been consistent during the analysis of many hundreds of plasma samples over a 2-year period. A more recent version of the ASTED system (XL) has operated to the same specification with increased sample throughput.

References

[1] M. Boolell, M.J. Allen, S.A. Ballard, S. Geti-Attee, G.J. Muirhead, A.M. Naylor, I.H. Osterloh, C. Jingell, Int. J. Impotence Res. 8 (1996) 47.
[2] K. Larsson, W. Hermann, P. Mooler, D. Sanchez, J. Chromatogr. 450 (1988) 71.
[3] R.A. Hall, T.P. Whitehead, J. Clin. Pathol. 23 (1970) 323.
[4] J.D.H. Cooper, D.C. Turnell, B. Green, F. Verillon, J. Chromatogr. 456 (1988) 53.
[5] M.M. Aerts, W.M.J. Beek, U.A.Th. Brinkman, J. Chromatogr. 500 (1990) 453.
[6] B. Green, J.D.H. Cooper, D.C. Turnell, Ann. Clin. Biochem. 26 (1989) 361.
[7] J.D.H. Cooper, D.C. Turnell, J. Chromatogr. 380 (1986) 109.
[8] J.D.H. Cooper, A.R. Buick, C.T.C. Fook Sheung, Lab. Robot. Autom. 6 (1994) 15.
[9] J.D.H. Cooper, C.T.C. Fook Sheung, A.R. Buick, J. Chromatogr.B 652 (1994) 15.