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Determination of eletriptan in plasma and saliva using automated sequential trace enrichment of dialysate and high-performance liquid chromatography

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Abstract

The use of the system, automated sequential trace enrichment of dialysates (ASTED), to prepare plasma and saliva prior to high pressure liquid chromatography of eletriptan (HPLC) is described. Chromatographic identification of one metabolite, UK-135,800 was also established. Using this technique the procedure was observed to be specific and linear over the range 0.50–250 ng/ml. The intra-batch imprecision (C.V.) of the method ranged from 0.56 to 5.70% at plasma eletriptan concentrations from 5.00 to 200 ng/ml, and the corresponding inter-batch imprecision ranged from 1.44 to 6.36%. At these plasma analyte concentrations, the overall inaccuracy (% bias) of the procedure ranged from −5.00 to 1.50%. Similar performances were observed for the estimation of eletriptan in saliva using near identical assay conditions. The application of the assay to a pharmacokinetic investigation during a clinical study is presented.

Keywords: ASTED; HPLC; Automated determination of eletriptan; Plasma; Saliva

1. Introduction

Eletriptan, [(*R*)-3-(1-methyl-2-pyrrolidinylmethyl)-5-[2-(phenylsulphonyl)ethyl]-1H-indole], is a potent specific 5-$HT_{1B/10}$ like receptor agonist undergoing development by Pfizer Ltd to improve the safety and efficacy of treatment for migraine. Following oral dosing of eletriptan several circulating metabolites have been characterised but none are considered to contribute significantly to the pharmacological activity. As a consequence a procedure was developed for the analysis of the parent compound alone. During its pharmacokinetic evaluation various biological matrices were required to be examined. As well as the development of a procedure for the determination of eletriptan in plasma an assay for the measurement of eletriptan in saliva is also reported.

Eletriptan (Fig. 1) has a basic functionality with a pK_a value of 9.2, although an acidic moiety is

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also present. A previous publication has demonstrated the use of the ASTED system to prepare basic compounds in plasma prior to HPLC separation [1] and has identified numerous problems associated with the analysis of such compounds. In particular non-specific binding of bases to all manner of vessel walls can have deleterious effects on many different assay types as well as on-line sample preparation systems like ASTED. Numerous applications of the ASTED system to a variety of biological matrices have previously been described in earlier publications [2–7]. This paper reports an ASTED/HPLC method for the estimation of eletriptan in plasma and saliva. The procedures incorporated the separation of a further compound, UK-136,509. This was added to aid assessment of sampling correctness during the preparation of samples in a completely automated manner prior to HPLC. Results obtained during validation of the ASTED/HPLC methods and its application to a pharmacokinetic study of the drug are presented.

2. Experimental

2.1. Instrumentation

Unless otherwise stated 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) model 306/5SC pump, a Gilson 118 UV detector and a Rheodyne 7010 (Cotati, CA) injection valve fitted on the ASTED unit. Control of the HPLC system, integration of chromatographic peaks and communication with the ASTED system (via Gilson Medical Electronics GSIOC) was made using a 715, V1.2, system controller and Microsoft Windows software V3.11 located in a Dell PC with Pentium processor.

2.1.2. Sample preparation (ASTED) unit

The ASTED XL unit (Fig. 2) comprised an auto-sampling injector; two 402 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 Rheodyne 7010 switching valve to isolate the dialyser unit from the trace enrichment device; 2 Kel F dialyser units (740 μl total donor volume), connected in series were used. The dialysers were fitted with 15 kDa cuprophan membranes (regenerated cellulose from Enka, Germany), and a stainless-steel trace enrichment cartridge (TEC, 4.6 × 5 mm) 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 was made using ASTED software (V2.00), Gilson Medical Electronics.

2.2. Reagents

2.2.1. General reagents

Unless otherwise stated, all chemicals were of an analytical grade obtained from Sigma (Poole,

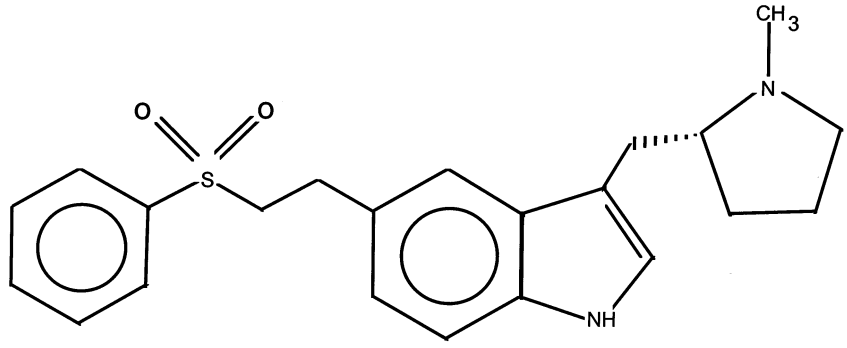

Fig. 1. Molecular structure of eletriptan.

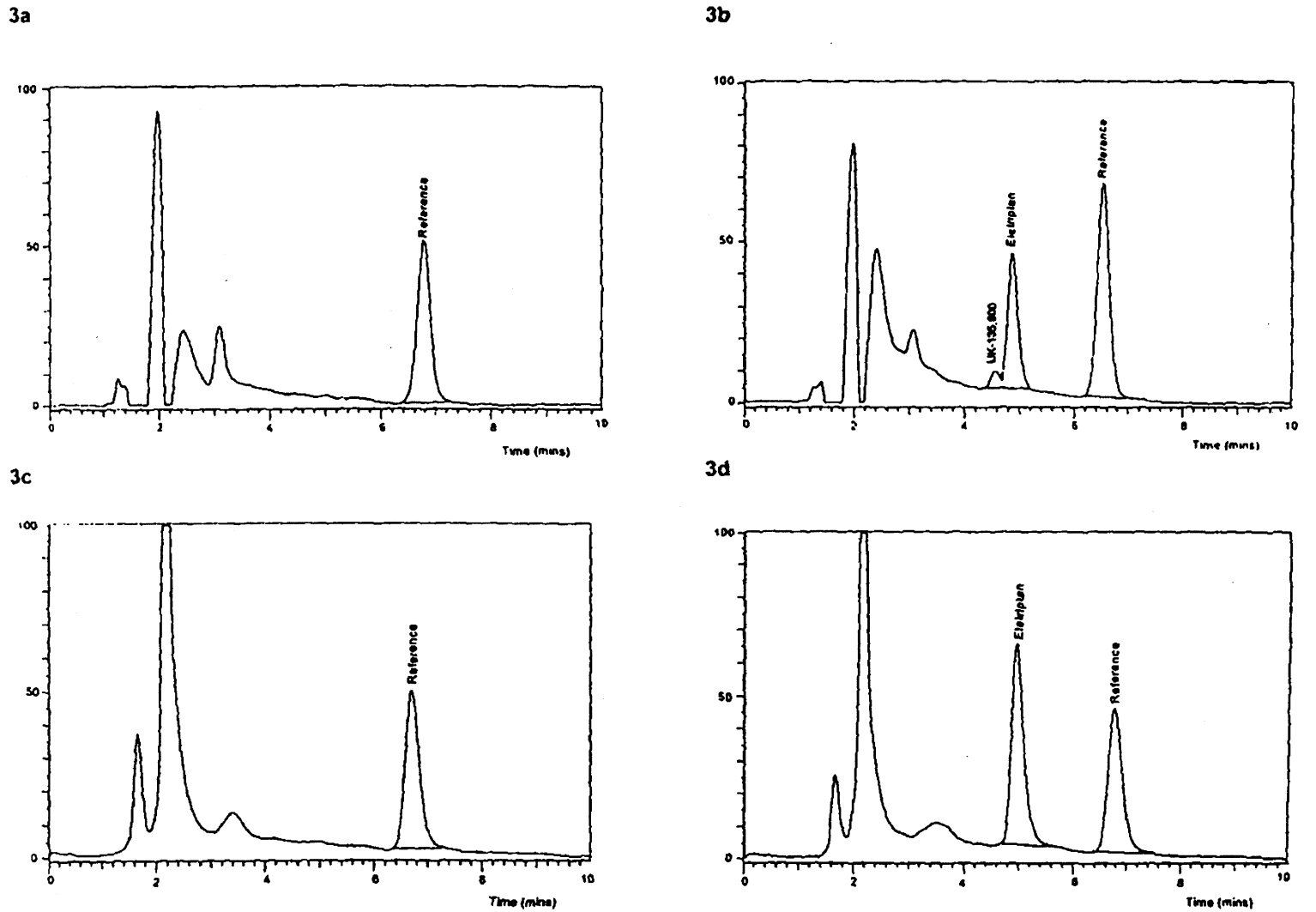

Fig. 2. Schematic diagram of the ASTED system used for the analysis of eletriptan. The arrows indicate the flow direction of reagents. Two dialyser units were connected in series to obtain the required assay sensitivity.

UK). HPLC grade water, prepared using a Purite (Thame, UK) system, was used for all reagent preparations. HPLC solvents were obtained from Romil (Cambridge, UK). The following reagents were prepared: Potassium hydrogen orthophosphate buffer (500 mmol/l, pH 3.5 and 7.0); 60% (v/v) acetonitrile in water; 10 mmol/l of potassium phosphate buffer (pH 7.0), solvent A, and 10 mmol/l potassium phosphate buffer (pH 7.0) containing 10% methanol (v/v), solvent B, were dispensed by syringes on the Gilson 402 dilutors on the donor and recipient side of the dialyser respectively. Solvent A enables location of sample in the donor channel of the dialyser whilst solvent B enables movement of dialysates to the enrichment cartridge; 1000 mmol/l monochloroacetic acid (MCA) was added to plasma prior to dialysis. For saliva, a 100 mmol/l MCA solution was used. Both MCA solutions contained 500 ng/ml of UK-136,509 as a reference compound.

2.2.2. Standard preparations

Eletriptan and its metabolite, UK-135,800 were supplied by Pfizer, Sandwich, Kent, UK. A stock solution containing 100 μg/ml of eletriptan (hydrobromide salt) was prepared in 20% (v/v) methanol/water. From this, working standards containing 0.025, 0.5 and 10 μg/ml of eletriptan were prepared in 20% (v/v) methanol/water. Six calibration standards ranging from 0.50 to 250 ng/ml of eletriptan for both plasma and saliva assays were prepared by supplementing appropriate blank matrices with the working standards. The concentration of eletriptan in each calibration standard was calculated as the free base.

2.3. Chromatographic conditions

An isocratic HPLC mobile phase was utilised comprising acetonitrile–potassium phosphate buffer (500 mmol/l, pH 3.5)–water (30:6:64 v/v/v) at a flow rate of 1.0 ml/min. Diethylamine hydrochloride (DEA), 20 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 C1 (Technicol, Stockport, UK). No guard column was employed and the analytical column was run at ambient temperature. The detector was set at 225 nm wavelength and an absorbance range of 0.001 AUFS for both plasma and saliva assays.

2.4. Sample preparation

Sample preparation was carried out in a completely automated manner using the ASTED XL system and optimised as follows: (1) For both plasma and saliva assays 570 μl of matrix were mixed with 130 μl of MCA/reference compound and 690 μl of this mixture located in the donor channel of the dialyser and remained static throughout the dialysis time period. (2) Trace enrichment was performed by moving 3500 μl of recipient solvent (in 500 μl pulsed volumes) through the TEC in a 4-min time period. (3) Following enrichment the donor tubing and dialyser channel were purged of sample with 1500 μl of donor solvent. (4) Using the ASTED robotics 200 μl of 60% acetonitrile–water followed by 800 μl of donor solvent was moved (via valve switching) through the TEC. (5) The Rheodyne high pressure valve was switched to the inject position and the analyte back flushed onto the HPLC column. (6) The system was then purged with 14 ml of donor and recipient solvent and the TEC regenerated with 200 μl of recipient solvent ready for the next sample.

2.5. Quantification

Calibration standards were located at the beginning and end of each analytical batch. A linear regression (weighting $1/X$ for plasma and $1/X^2$ for saliva) was performed on the peak areas and concentrations of eletriptan. These weighting factors improved the performance of the assay towards the low end of the calibration curve. The regression lines established were used to calculate test analyte concentrations by interpolation. The automatic addition of the reference compound (UK-136,509) was included to indicate any incorrect sampling and not for quantification purposes.

Table 1
Intra- and inter-assay imprecision and inaccuracy of the ASTED-HPLC method for the analysis of eletriptan in plasma and saliva

Matrix	Spiked eletriptan 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 (%)
Plasma	0.50	0.54	15.6	8.00	–	–	–
	5.00	4.98	5.70	−0.42	4.99	6.36	−0.20
	20.0	19.1	4.22	−4.72	19.3	4.26	−3.50
	80.0	81.7	0.79	2.12	81.2	1.44	1.50
	200	192	0.56	−3.87	190	2.20	−5.00
Saliva	0.50	0.49	11.2	−2.00	–	–	–
	3.00	2.84	2.46	−5.33	2.71	4.80	−9.67
	125	126	0.60	0.80	125	1.80	0.00
	200	194	0.73	−3.00	195	2.22	−2.50

2.6. Control samples

Depending on the matrix under investigation drug-free matrices were supplemented with a varying range of eletriptan concentrations (Table 1). These samples were aliquoted and stored at −20°C. The eletriptan methanol–water (20% v/v) solutions used to supplement the plasma were prepared from separate weighings to those solutions used to prepare the calibration standards.

3. Results

3.1. Optimisation of chromatography and sample preparation conditions

3.1.1. Chromatography conditions

An elution pH of 3.5 was used for the separation of eletriptan and the reference compound, UK-136,509 (Fig. 3b,d) for the matrices under investigation. The inclusion in the mobile phase of a silanol blocking amine (DEA) together with the use of Kromasil column C1 packing material ensured symmetrical peak shapes of the compounds under investigation.

3.1.2. Sample preparation conditions

For both plasma and saliva matrices, two dialyser units connected in series were required to obtain sufficient sensitivity for estimation of the compounds at the lower limit of quantification (i.e. 0.50 ng/ml). Sample to sample interactions were minimised by purging the ASTED donor fluid lines with phosphate buffer (pH 7.0, 10 mmol/l). 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

Using the regression weighting described the response varied linearly over the analytical range employed (0.50–250 ng/ml for both plasma and saliva). The lower quantification limit was set at the lowest standard concentration on the calibration curve for both matrices.

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 and saliva eletriptan concentrations over four separate analytical batches. Only intra-batch imprecision was examined at the lower limit of quantification for both assays. The results for both plasma and saliva matrices are shown in Table 1 together with the intra-batch imprecision data from one of the analytical batches.

3.2.3. Assay specificity

For both the matrices tested, drug-free material from six volunteers were assayed using the procedures described. No chromatographic interferences were observed as typified by a chromatogram from the preparation of a blank sample of each matrix (Fig. 3). Solutions of sumatriptan, nifedipine, indomethacin, paracetamol and propranolol were also analysed 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 spiked with 80 ng/ml of eletriptan and blank saliva spiked with 125 ng/ml of eletriptan using methanol–aqueous standards of eletriptan to calibrate the assay, was estimated to be 97.6% (1.74) and 105% (1.80) respectively. Further to this, six different plasma samples were each spiked with 80 ng/ml of eletriptan and analysed using plasma standards to calibrate the assay. The accuracy (C.V. %) of the assay using this experimental model was determined to be 106% (2.34). The data for each sample type indicates that matrix effects do not affect inter-assay imprecision.

3.2.5. Instability of the analytes

Using the ASTED/HPLC assay procedure, no obvious degradation of eletriptan in plasma or saliva occurred over 24 h at room temperature or after three freeze/thaw cycles. No obvious degradation of eletriptan was observed over 6 months in saliva and 12 months in plasma when stored at −20°C. Similar investigations were conducted for aqueous solutions of the analytes stored in standard polystyrene vials and in glass. Approximately 40% losses of eletriptan were observed after storage in plastic over 1 month at room

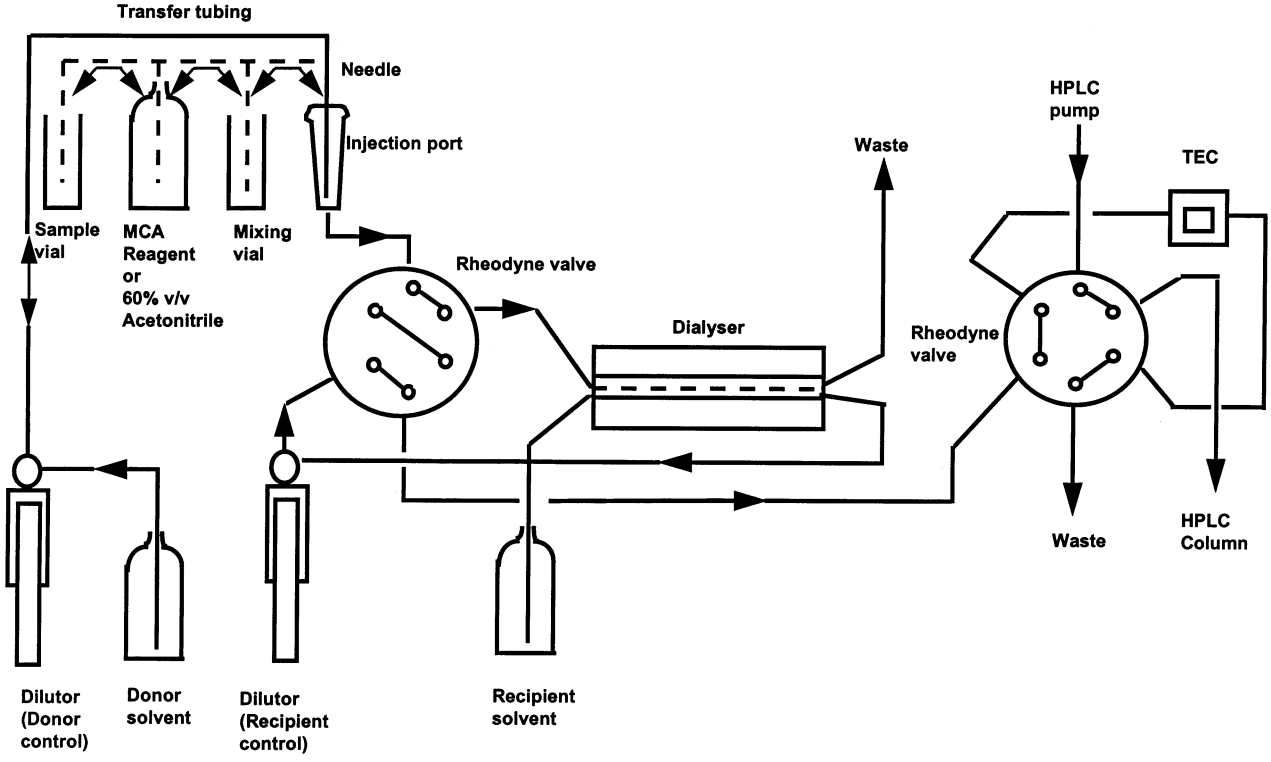

Fig. 3. Chromatograms of (a) blank plasma; (b) plasma from a subject with a measured eletriptan concentration of 40.3 ng/ml; (c) blank saliva; (d) saliva from a subject with a measured eletriptan concentration of 68.6 ng/ml.

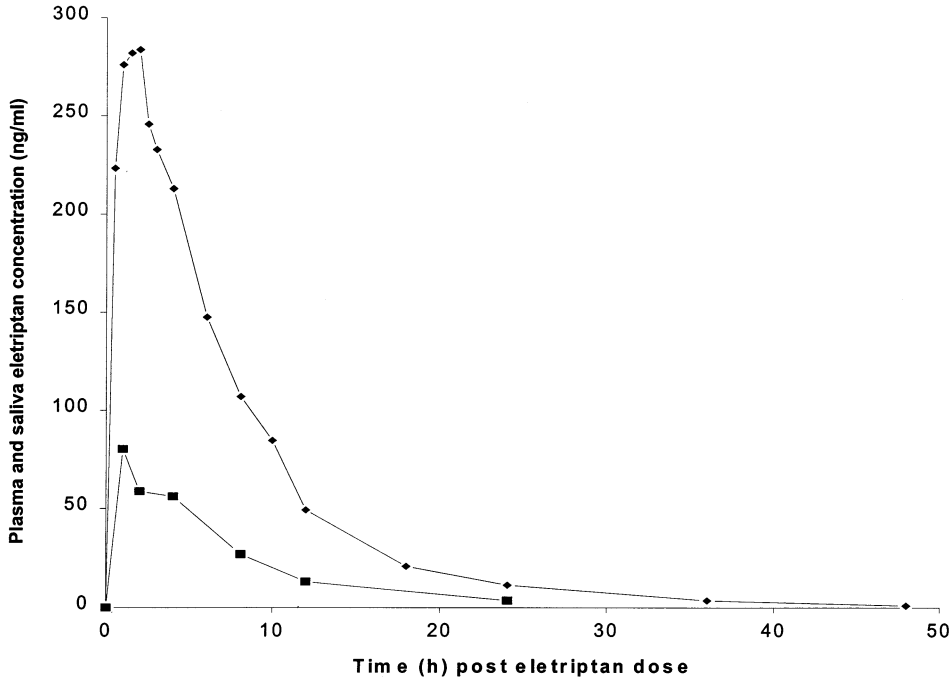

Fig. 4. Pharmacokinetic plot of plasma (◆) and saliva (■) eletriptan concentrations from a subject receiving a single oral 120-mg dose of eletriptan during a randomised, double-blind, placebo controlled study of escalating single oral doses of eletriptan.

temperature compared with negligible losses in glass. Such losses in plastic could be negated by including 20% (v/v) methanol in the aqueous solution.

3.2.6. Sample carry-over

Carry-over was determined by analysing a blank sample of each matrix type following the assay of the calibration standard with the highest eletriptan concentration. This was performed in triplicate and the carry-over estimated to be $<$ 0.5% in each case.

3.2.7. Pharmacokinetic investigations

Biological samples from numerous phase I, II and III clinical studies have been assayed using the ASTED/HPLC procedure. As an example the application of the method to the analysis of plasma and saliva samples from a subject receiving 120 mg of eletriptan as a single oral dose, during a randomised, double-blind, placebo controlled study of escalating single oral doses of eletriptan is presented. Blood and saliva samples were collected pre-dose and at intervals up to 48 and 24 h post-dose respectively. Plasma and saliva were stored at -20°C until analysis. The pharmacokinetic profile from the analyses is shown in Fig. 4.

4. Discussion

Numerous publications concerning the potential of the on-line ASTED system have been presented [1–7]. This paper further amplifies its diversity in that the system is capable of handling a variety of biological matrices. Other matrices have been investigated and validated methods developed for the analysis of eletriptan. These include application of the ASTED system to the analysis of human breast milk and urine matrices. The use of Cartesian *xyz* robotics can readily be adapted to a wide variety of applications in an automated manner and coupled with the incorporation of ASTED for sample clean-up, highly specific assays are feasible even with the most complex of sample matrices.

4.1. Chromatography

Following validation of the assay initial clinical studies showed the emergence of the eletriptan metabolite, UK-135,800, in plasma. The adoption of a mobile phase of pH 3.5 was sufficient to give 95% resolution of the eletriptan metabolite, UK-135 800 (Fig. 3b), when analysing plasma. This incomplete resolution has been shown not to affect the accuracy of the plasma assay, particularly at low eletriptan concentrations. UK-135 800 was less evident when analysing saliva samples (Fig. 3d). More recently complete resolution of the metabolite and its the parent compound has been achieved using a mobile phase containing a potassium phosphate buffer of pH 6.5. However all data for pharmacokinetic investigations has been gathered using the validated assay presented and mobile phase conditions identified.

4.2. ASTED sample preparation

4.2.1. Non-specific binding of bases during sample preparation

Losses of bases stored in plastic containers can be attributed to non-specific binding of the compounds to plastic surfaces [1]. This was evident in the case of eletriptan where losses were observed during storage in polystyrene as opposed to the negligible losses following storage in glass. Losses may also accrue during passage of liquids through plastic transfer tubing. These difficulties are generally overcome when analytes are retained in plasma especially when moderate or relatively high protein binding occurs as is the case for eletriptan ($>76\%$, unpublished data). However the problems of solution storage in vials can be amplified in the case of automation. Like the previous publication describing the ASTED procedure for the analysis of bases [1] automating procedures using Cartesian *xyz* robots suffer from the major disadvantage that all sample/liquid movements pass through single line transfer tubing. Excessive sample to sample interactions may occur due to protein build-up on plastic surfaces and the binding of bases to both protein and exposed plastic binding sites. Likewise binding of compounds to cuprophan dialysis membranes has also been observed [8] although this can be overcome with the incorporation of organic solvents in, for example, the recipient solution. 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 transfer lines with a phosphate buffer (pH 7.0, 10 mmol/l).

4.2.2. Optimisation of the ASTED parameters

4.2.2.1. Dialysis parameters. Earlier publications [1,2] have clearly identified 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. Although the rates of transfer are increased, dilute analyte concentrations in the dialysate occur and enrichment is usually required. Combining the two processes, i.e. membrane clean-up and trace enrichment is fundamental for both to succeed. For eletriptan the lower quantification limits of 0.5 ng/ml in plasma and saliva were desirable and to achieve this, two large dialyser units connected in series were required with a total donor capacity of approximately 740 μl. Target lower quantification limits (0.5 ng/ml) could be achieved with the analysis of 570-μl sample volumes due to the efficiency of the sample preparation process producing chromatographic separations with few background interferences (Fig. 3a,c).

4.2.2.2. Trace enrichment parameters. The affinity of a compound for stationary phase is measured by its breakthrough volume [9]. Affinities are governed by a variety of reactions and in the case of this assay enrichment occurs due to ion exchange mechanisms between the positively charged amine moiety of eletriptan and the exposed negatively charged silanols on the non-end capped Hypersil C1 enrichment material. Good assay specificity is achieved by washing the enriched compounds with organic solvents (e.g. acetonitrile) to remove non-polar interferences. In this assay, 200 μl of 60% (v/v) acetonitrile–water gave sufficient clean-up without reducing the breakthrough volume of eletriptan to such levels

that losses occurred. In agreement with a previous communication [1] the concentration of acetonitrile used depends on the basicity of the molecules under investigation. 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. A 10 mmol/l phosphate solution (pH 7.0) was sufficient in this procedure to buffer reduced MCA concentration in the dialysate and enrichment of the dialysate at pH 7.0 avoided chromatographic interferences.

4.2.2.3. Sample preparation and matrix effects. Previous discussions [1,2] concerning between sample matrix variations have identified that these variances can have disastrous effects on assay performance for many biological assay procedures especially when dealing with highly protein bound compounds. For measurement of total drug concentrations, elimination of protein binding is essential especially when membranes are involved in the preparation of plasma type matrices. Altering the pH of plasma using MCA has been shown to be beneficial for basic molecules such as xanthines and sildenafil [1,2] and this proved to be the case for eletriptan. The actual concentration of MCA used depends on the protein concentrations present in the matrix under investigation. In the case of saliva samples, much reduced MCA concentrations were employed compared with those used for a plasma matrix. 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. To some extent this is confirmed by the recovery data obtained when supplementing six different plasma samples with eletriptan.

4.3. Application to pharmacokinetic investigations

The method described has been successfully applied to the measurement of eletriptan plasma and saliva concentrations in samples from many pharmacokinetic studies involving young healthy subjects, special patient groups (hepatic and renal impairment) and elderly healthy subjects receiving oral eletriptan. More recently the measurement of eletriptan in saliva has been applied to a dose escalation study and population kinetic studies. Although saliva may not be ideal as a matrix due to the variability of excretion flow and collection [10,11] it has the advantage of offering non-invasive investigations of the drug in subjects suffering from acute migraine attacks. Example pharmacokinetic profiles are presented for plasma and saliva using the ASTED assay procedure 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.

4.4. Assay performance

The ASTED procedure has been shown to be highly selective (Fig. 3a,c) for the compounds under investigation using HPLC as the means to separate and quantify the eletriptan. Using the ASTED and HPLC assay conditions described, Gaussian peak shapes were observed (Fig. 3b,d) and several thousand injections of prepared plasma and saliva samples were achieved without extensive loss of column efficiencies. Combined with this several hundred injections of prepared plasma and saliva 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 and the data obtained (Table 1) indicate that the procedure was accurate. The use of this technique has provided a rapid means for the analysis of eletriptan and the acceptable assay performance has been consistent during the analysis of many hundreds of plasma and saliva samples over a 4-year period.

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