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DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC ASSAY FOR THE DETERMINATION OF SUMATRIPTAN IN PLASMA

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SUMMARY

Sumatriptan succinate is a novel compound currently in development for the acute treatment of migraine During early studies in man a sensitive and selective assay was required, which had to be developed rapidly, to determine plasma concentrations following an intravenous infusion Thermospray liquid chromatography–mass spectrometry combined with the advanced automated sample processor was selected to achieve this Although the assay was required quickly criteria for intra- and inter-assay accuracies and precisions of ± 10% had to be achieved These were obtained only by using a co-eluting deuterium-labelled internal standard Attempts to use a homologue as an internal standard, which did not co-elute with sumatriptan, gave inferior results The assay was linear over the calibration range 2–50 ng/ml with a limit of quantification of 2 ng/ml The application of the technique to the analysis of samples from a volunteer study is demonstrated

INTRODUCTION

During the early stages of drug development, bioanalytical assays are required to provide pharmacokinetic data in animals and in man. It is accepted that some procedures such as radioimmunoassay, for example, may have a long development time. Thus there is a need for rapidly developed interim methods to provide the early information on drug absorption Whilst these assay procedures may not be the final choice for routine analysis, nonetheless they should be robust and fulfil acceptable criteria for sensitivity and reproducibility.

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Thermospray liquid chromatography–mass spectrometry (TSP LC–MS) offers several advantages for the rapid development of such assays. The development of derivatisation procedures, generally a requirement for the determination of polar drugs by gas chromatographic procedure, is time-consuming; it is, however, often unnecessary for LC analyses. In addition, the selection of the mass spectrometer as a detector in the first instance rather than UV, fluorescence or electrochemical detectors gives the advantage of mass specificity This can reduce the time for development of a suitable extraction procedure. The thermospray technique [1,2] provides a reliable and robust interface which is capable of handling reversed-phase chromatographic eluents with a high aqueous content and a flow-rate of 1–2 ml/min, that are commonly used for the quantitative analysis of polar drugs in biological matrices. Furthermore, the technique can be automated by combination with the advanced automated sample processor (AASP) [3] or an autoinjector [4].

We have already demonstrated the feasibility of the combination of the AASP with TSP LC–MS in an evaluation exercise for the analysis of an established product, labetalol, in human plasma [3]. The importance and the role of the combined technique are described in this paper for the rapid development of a fully validated assay for a novel compound sumatriptan succinate, 3-[-2-(dimethylamino)ethyl]-N-methyl-1H-indole-5-methanesulphonamide succinate[a], in human plasma. Sumatriptan succinate is a 5-hydroxytryptamine receptor ($5HT_1$-like) agonist currently in development for the acute treatment of migraine [5]. An assay was required for this compound having a limit of quantification of 5 ng/ml and accuracy and precision of $\pm 10\%$ to determine plasma concentrations in man following administration by intravenous infusion

EXPERIMENTAL

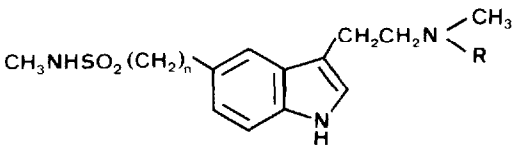
Reagents and materials

All reagents were of analytical grade. Sumatriptan (I) (succinate salt), the higher homologue (II), [$^2H_3$]sumatriptan (III) and [^{14}C]sumatriptan (IV) (succinate salt) (Fig. 1) of specific activity 1 3 MBq/mg base were synthesised in the Chemistry Division, Glaxo Group Research (Ware, U.K) [$^2H_3$]Sumatriptan contained less than 0.1% unlabelled sumatriptan.

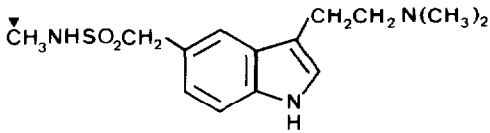
TSP LC–MS parameters

The instrumentation used in this study has been described previously [4] Two chromatographic systems were used. In system A, a 5-μm Partisphere C_{18} column of dimensions 110 mm$\times$4.6 mm I D. (Whatman, Maidstone, U.K.) was eluted with methanol–0 1 *M* ammonium acetate (60 40) at a flow-rate of

[a]Sumatriptan succinate is the approved name for GR 43175C



I n = 1, R = CH_3
II n = 2, R = CH_3
III n = 1, R = C^2H_3



IV

Fig 1 Structures of compounds I–IV, ▼ denotes the position of the ^{14}C label

1 ml/min. System B differed in that a 50 mm×4.6 mm I.D. stainless-steel column packed with 3-μm Spherisorb ODS-2 particles (Chromatography Services, Wirral, U K.) and protected by a Waters C_{18} Guard-Pak pre-column module (Waters Chromatography, Harrow, U.K.) was used The ion source tuning was optimised using the solvent cluster ions An interface tip temperature of 148–152°C and an ion source temperature of 250°C were used. Data were acquired and processed using the standard software supplied by Hewlett-Packard.

Quantitative analysis

Preparation of standards Standards used for both calibration and for assay validation were prepared in control human plasma. Any standards not analysed immediately after preparation were stored frozen at −20°C. Plasma samples taken from subjects following administration of sumatriptan succinate were stored at −20°C prior to analysis.

Extraction and analyses AASP C_2 cartridges (Jones Chromatography, Llanbradach, U.K.) were conditioned with methanol (1.8 ml) followed by distilled water (1 8 ml) using a Vac-Elut module (Varian, Walton-on-Thames, U K.) operated at ca. 4 bar air pressure Standards and plasma samples from the clinical study were centrifuged to remove any particulate material, then aliquots (1 ml) of the supernatant were pipetted into 2-ml vials. The internal standard, either II (24 ng) or III (50 ng), was added to each and the contents were vortex-mixed These were each transferred to the appropriate reservoirs on the Vac-Elut module for elution through the cartridges. A modification to the assay introduced prior to full assay validation was to use a Hamilton Microlab M diluter–dispenser (V.A. Howe, London, U K.) to dispense the internal standard solution followed by the calibration standard or sample directly into the cartridge reservoir. Each cartridge was washed with distilled water (1 8 ml) followed by water–methanol (7 3, 1 8 ml). The cassettes were loaded

onto the AASP module for TSP LC–MS analysis. The AASP run time was 4 min per cartridge.

Under selected ion recording (SIR) the abundances of the protonated molecular ions, *m/z* 296 derived from sumatriptan and either *m/z* 310 or *m/z* 299 derived from the homologue II or [$^2H_3$]sumatriptan, respectively, were each monitored sequentially for 350 ms. The chromatograms were smoothed before integration. A calibration line was constructed for each series of analyses from a plot of the ratio of the peak heights of the ions derived from sumatriptan and its internal standard versus the concentration of sumatriptan using a least-squares linear regression analysis. Concentrations of sumatriptan were determined from this calibration line.

Extraction efficiency The extraction efficiency was determined in quadruplicate at two concentrations of [^{14}C]sumatriptan, 10 and 40 ng/ml of plasma. Aliquots of each standard (1 ml) were applied to pre-treated C_2 AASP cartridges. The cartridges were washed with water (1.8 ml), then eluted with methanol–0.1 *M* ammonium acetate (60 40, 1.8 ml). In a separate experiment the water wash was followed by a water–methanol (7 3, 1.8 ml) wash prior to elution. The eluate was added to Pico-fluor 30 liquid scintillation cocktail (Packard Instrument, Caversham, U.K.) (16 ml) and the radioactive content was determined in a Tracor Analytic Mark III liquid scintillation counter (Tracor Analytic, Elk Grove Village, IL, U S.A.). The external standard channels ratio method was used for the determination of counting efficiency. Two portions (1.0 ml) of the original plasma solution were added to Pico-fluor 30 scintillant. The extraction efficiency was determined by reference to these results.

RESULTS AND DISCUSSION

TSP LC–MS analysis of sumatriptan

Full-scan TSP mass spectral data are easily achievable on 10 ng of sumatriptan, the spectrum showing, as base peak, the protonated molecular ion at *m/z* 296. (Fig. 2a) On SIR of *m/z* 296, 500 pg injected onto the column was detectable (Fig. 2b). The limit of detection was 100 pg (signal-to-noise ratio of 3 1).

Assay development and validation

A semi-automated solid-phase extraction procedure using the C_2 cartridges available for the AASP was developed. This has the advantage that the whole of the extract of 1 ml of plasma can be eluted from the cartridge onto the analytical column, thus maximising sensitivity. The efficiency of the isolation method was precise when determined using [^{14}C]sumatriptan (40 ng/ml, $94.0 \pm 1.6\%$, $n=4$; 10 ng/ml, 93.4 ± 1 3%, $n=4$). No significant decrease in extraction efficiency was obtained when using an additional water–methanol cartridge wash step.

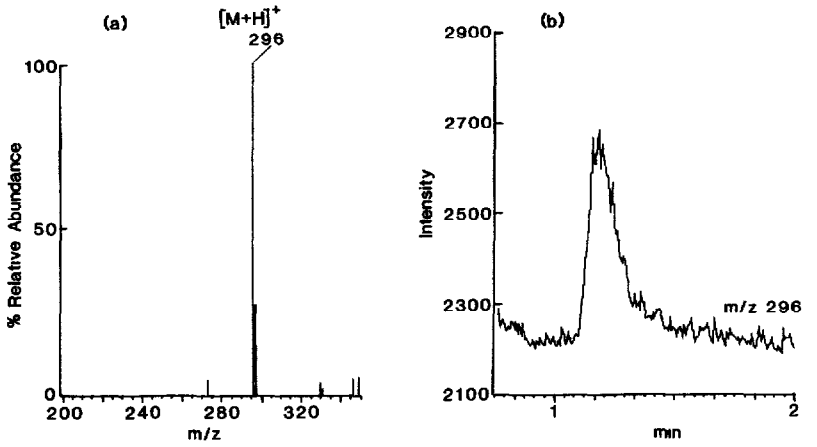


Fig 2 (a) TSP mass spectrum of 10 ng sumatriptan, (b) SIR chromatogram of 500 pg sumatriptan These were obtained using chromatographic system B

An internal standard was needed to compensate for run-to-run variability in the efficiency of the TSP ionisation process. During assay development two internal standards were evaluated, initially the homologue II and at a later stage [$^2H_3$]sumatriptan. The TSP mass spectra of both compounds showed protonated molecular ions at *m/z* 310 (II) and *m/z* 299 ([$^2H_3$]sumatriptan) with no observable fragmentation.

Under the chromatographic conditions of system A, sumatriptan was partially resolved from II with retention times of 2.1 and 2 3 min, respectively. With this internal standard, the quality of the calibration data obtained over the range 3–40 ng/ml of plasma was variable, and the intra- and inter-assay variabilities were too high to meet the criterion of 10% set for the method. For example, at a nominal concentration of 22 ng/ml of plasma, mean concentrations of 22.5 ng/ml ($n=6$) and 21.4 ng/ml ($n=5$) were determined on successive occasions with coefficients of variation of 7 and 26%, respectively. At a nominal concentration of 6 ng/ml the mean determined was 7.4 ng/ml ($n=5$) with a coefficient of variation of 15%. In contrast, the use of [$^2H_3$]sumatriptan as an internal standard, which co-eluted with sumatriptan, gave significantly improved results for both linearity of calibration and precision and accuracy

The calibration graphs relating the ratio of the peak heights of *m/z* 296 to *m/z* 299 with the concentration of sumatriptan in prepared standards were linear in the range 2–50 ng/ml of plasma. The regression characteristics were typically slope=0.021, intercept=0 004, with a correlation coefficient (r) of 0.9995. Over the range 5–50 ng/ml of plasma, the intra-assay precision was within 5% and at 2 ng/ml it was 11% (Table I). The accuracy, which was defined as the percentage difference between the mean concentration found

TABLE I

INTRA-ASSAY VARIABILITY WITH [$^2H_3$]SUMATRIPTAN (50 ng/ml OF PLASMA) AS INTERNAL STANDARD

Analyses carried out using chromatographic system B

Nominal concentration (ng/ml)	Concentration found (mean ± S D , $n=6$) (ng/ml)	Accuracy[a] (%)	Coefficient of variation (%)
0	Not detectable	–	–
2	1 8±0 2	10 0	11 1
5	4 7±0 2	6 0	4 3
10	9 7±0 3	3 0	3 1
20	19 9±0 5	0 5	2 5
30	29 1±0 6	3 0	2 1
50	48 5±1 5	3 0	3 1

[a]Defined as the percentage difference between the mean concentration found and the theoretical concentration

TABLE II

INTER-ASSAY VARIABILITY WITH [$^2H_3$]SUMATRIPTAN (50 ng/ml OF PLASMA) AS INTERNAL STANDARD

Analyses carried out using chromatographic system B

Nominal concentration (ng/ml)	Concentration found (ng/ml)				Accuracy[a] (%)	Coefficient of variation (%)
	Assay 1	Assay 2	Assay 3	Mean		
3 9	4 1	3 8	3 6	3 9	0	10 2
	4 4	–[b]	3 4			
20 6	21 0	20 4	19 7	20 0	2 9	3 6
	20 5	19 0	19 6			
41 2	40 3	37 0	40 5	38 7	6 1	3 9
	39 1	37 7	36 8			

[a] Defined as the percentage difference between the mean concentration found and the theoretical concentration

[b] Sample lost during analysis

and the theoretical concentration, was 10% or better (Table I). Similar results were obtained for the inter-assay variability (Table II)

Although the chromatographic conditions were changed slightly for the analyses with the two internal standards, this is not thought to be the reason for the wide difference in assay performance. This difference may result from a combination of factors. Signal instability is commonly observed in TSP LC–MS analyses [6]. This can arise from instrumental changes occurring both within an analytical run and between analyses. Consequently an internal standard which does not co-elute with the component of interest may not compensate adequately for instrumental variability. Furthermore the composition of

the biological extract eluting from the AASP C_2 cartridge is complex, hence a number of components are likely to elute with sumatriptan and the homologue II Competitive ionisation of these compounds may result in changes in the ionisation efficiencies of the compounds of interest. If there is a run-to-run variation between analyses in the proportions of these components present, there will subsequently be a variation in the ionisation efficiency of sumatriptan and II. In addition the two compounds do not co-elute and may be affected to different extents This might be overcome by improving the cleanliness of the biological extract or as in this case by using a co-eluting internal standard.

The limit of quantification for the assay was set at 2 ng/ml Although sequential analysis of 500 pg/ml standards could be determined with an acceptable coefficient of variation, the precision could not be maintained throughout a typical batch size of fifty analyses. A component, also yielding an ion at *m/z* 296, eluted from the AASP cartridge onto the analytical column. Its retention time was approximately 28 min and it co-eluted with sumatriptan in later analyses. The contribution to the peak height of *m/z* 296 from this component was

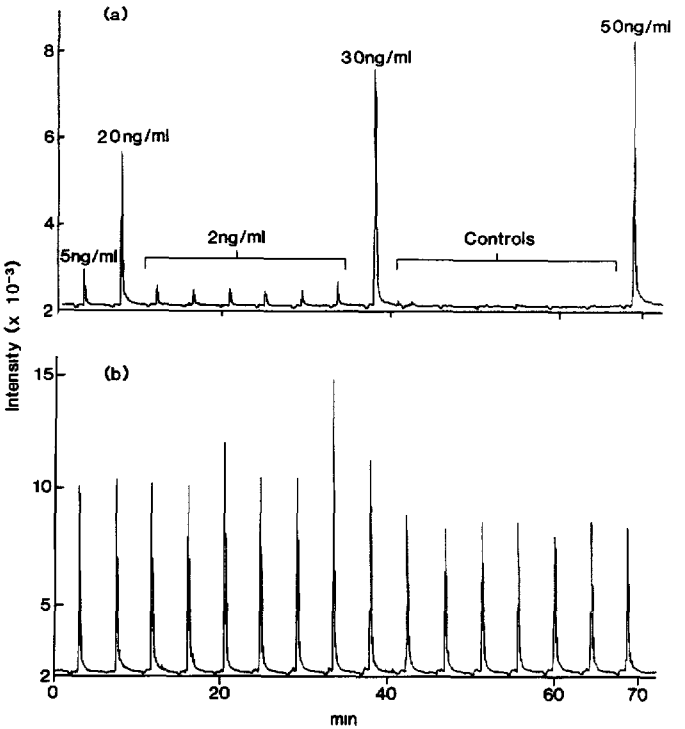


Fig 3 SIR chromatograms derived from the sequential analysis of calibration and replicate standards in plasma from prepared AASP cartridges using chromatographic system B (a) *m/z* 296, $[M+H]^+$, derived from sumatriptan, (b) *m/z* 299, $[M+H]^+$, derived from 50 ng $[^2H_3]$sumatriptan per ml of plasma

approximately 20% at a concentration of 500 pg/ml Attempts to remove the contaminant by pre-washing the cartridge were unsuccessful and its presence limited the assay to 2 ng/ml, where the contribution from the interfering component was negligible. Fig. 3 shows the SIR chromatograms for *m/z* 296 and 299 during the sequential analysis of sixteen prepared AASP cartridges. These results were obtained during method validation and are derived from the analysis of calibration and replicate standards prepared from plasma. The need for an internal standard is shown very effectively by the wide variation in peak heights which is observed over the sixteen analyses for *m/z* 299 derived from [$^2H_3$]sumatriptan (Fig. 3b).

Sample analysis

This assay has been used to provide preliminary data on plasma concentrations following administration of sumatriptan succinate to man. Fig. 4 shows the SIR chromatograms derived from a plasma sample taken prior to administration of 64 μg sumatriptan base per kg of body weight by intravenous infusion and a sample taken 2 h later. The SIR chromatogram of the control sample (Fig. 4a) demonstrates the mass specifity of the technique in showing the absence of interference from co-eluting components derived from plasma. Fig. 4b obtained from the 2-h plasma sample shows the response equivalent to a concentration of 6.1 ng/ml Pharmacokinetic data derived from the plasma concentration versus time profiles (Fig. 5) for two subjects, each receiving 64

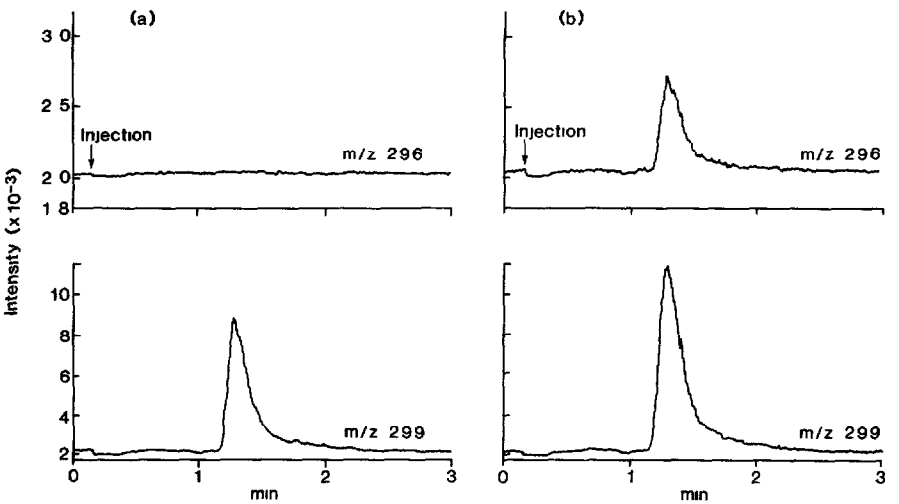


Fig 4 SIR chromatograms derived from (a) a pre-dose plasma sample and (b) a 2-h sample containing 6 1 ng sumatriptan per ml of plasma from a subject who received 64 μg sumatriptan base per kg of body weight by intravenous infusion

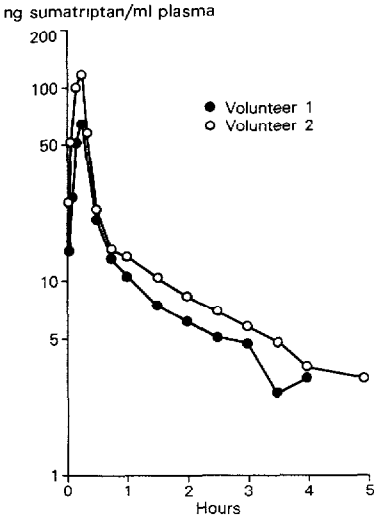


Fig 5 Plasma concentration versus time profiles for two subjects during and following cessation of a 15-min intravenous infusion of sumatriptan succinate at a dose level of 64 μg sumatriptan base per kg of body weight

μg sumatriptan base per kg of body weight by intravenous infusion, were used to define further clinical studies.

CONCLUSION

A robust, reliable and sensitive assay using the AASP combined with TSP LC–MS was developed and fully validated for the determination of sumatriptan in human plasma. To fulfil the requirement for an intra- and inter-assay accuracy and precision of $\pm 10\%$ or better, it was necessary to use a co-eluting internal standard, i e. [$^2H_3$]sumatriptan. The precision measurements obtained using the homologue II, which did not co-elute, were more variable. However, had the assay requirements been less stringent, the method using II would have been adequate for providing approximate concentrations The assay was developed in approximately six weeks, thus demonstrating an effective role for TSP LC–MS in the rapid development of sensitive and selective assays.

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