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Determination of the two major human metabolites of tipredane in human urine by high-performance liquid chromatography with column switching

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Abstract

An automated method based on column-switching reversed-phase in high-performance liquid chromatography the heart-cutting mode has been developed for the simultaneous determination of the two major human metabolites of tipredane, FPL 66365XX and FPL 66366XX, in human urine. The limit of quantification of the method was 25 ng/ml for both analytes from a urine injection volume of 100 μl. The intra- and inter-assay precision and accuracy were acceptable between 25 and 5000 ng/ml. No significant interferences were observed from either tipredane or a selection of its putative metabolites, or urine constituents in samples from male and female volunteers. Both analytes were found to be stable in human urine when stored at room temperature for two days, at 4°C for six days, in a freezer at or below −20°C for three weeks, and when the urine samples were subjected to three freeze–thaw cycles The method was unusual in that the initial separation was performed on a non-polar, octadecylsilane, column and the final separation on a more polar, trimethylsilane column. These columns were selected only after the investigation of a wide range of reversed-phase columns. The method's success was based on the greatly differing selectivities shown towards the two analytes by the organic modifiers, methanol and acetonitrile, present in the mobile phases used for the extraction and analytical stages

Keywords: Tipredane

1. Introduction

Tipredane [(11β,α-17-(ethylthio)-9α-fluoro-11β-hydroxy-17-(methylthio)androstra-1,4-dien-3-one, Fig. 1] is a potent, topically active, synthetic glucocorticoid developed by E. R. Squibb and Sons for the treatment of inflammatory skin diseases [1]. The compound was investigated by Fisons as a treatment for bronchial asthma and related disorders.

After administration in man the drug undergoes rapid and complex metabolism [2,3]; two major metabolites, FPL 66365XX (I, Fig. 1) and FPL 66366XX (II, Fig. 1) are excreted in urine [4]. During the clinical programme for tipredane, volunteers received low doses of tipredane and con-

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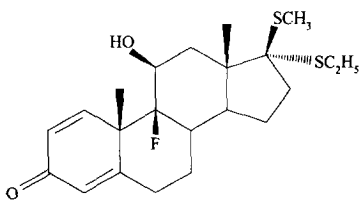
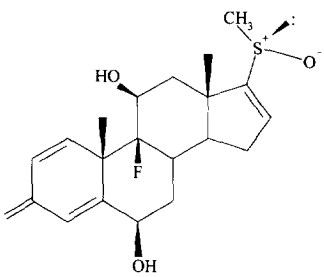
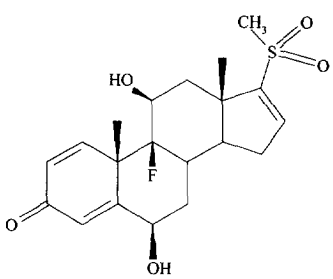


Tipredane



I



II

Fig. 1. Structures of tipredane, FPL 66365XX (I) and FPL 66366XX (II).

centrations of the parent drug at sub ng/ml concentrations were observed in plasma by radioimmunoassay [5,6]. A method to determine these two metabolites in human urine at concentrations as low as 25 ng/ml was required to support clinical studies. The available methods involved solid-phase extraction prior to analysis by high-performance liquid chromatography (HPLC) and were neither robust nor sufficiently sensitive. As column-switching was successful in the determination of the major male rat metabolite in rat urine [7] its use in the determination of I and II was investigated.

Urine samples were directly injected into the HPLC system, after minimal pre-treatment, thus avoiding manual sample extraction. Two reversed-phase HPLC columns were used in a heart cutting column switching configuration. The portion of the column eluate from the extraction column (Hypersil ODS) containing the analytes was switched, via an injection loop, to the analytical column (Zorbax TMS) where the final separation was carried out.

The usual criteria for the extraction stage of such a heart-cutting column-switching system are the separation of the analyte from the bulk of the urine background and its elution within a short retention time (5–10 min) and with a narrow peak width (ca. 1 min). Here, there were the additional requirements for the analytes to co-elute on the extraction column but separate on the analytical column, to enable the simultaneous analysis of both metabolites. Prior to the selection of Hypersil ODS as the extraction column and Zorbax TMS as the analytical column, many other stationary phases based on cyanopropyl (CN), trimethylsilane (C_1), phenylsilane, octylsilane (C_8) and octadecylsilane (C_{18}) were investigated as extraction and analytical columns. The finalised method was unusual in that it used a supposedly more polar TMS (C_1) column for the final separation and a less polar ODS (C_{18}) column for the initial separation.

The properties of the assay and its use in support of a clinical study are described here. The outcome of coupling other columns together is also described here to exemplify the flexibility of this mode of column-switching.

2. Experimental

2.1. Materials

Tipredane was supplied by Bristol Myers Squibb (Sword Laboratories, Dublin, Ireland). The metabolites of tipredane were products of Fisons, Pharmaceutical Division (Loughborough, UK).

Methanol was of ultra-gradient grade and obtained from Romil Chemicals (Shepshed, UK). Water was purified by distillation and passage through a Waters Milli-Q Plus Water Purification System supplied by

Millipore (UK) (Waters Chromatography Division, Harrow, UK). The rest of the HPLC reagents were of HPLC grade and were purchased from Fisons Scientific Equipment (Loughborough, UK).

Blank human urine samples were obtained from a panel of healthy human donors with no exposure to tipredane, and stored at or below −20°C. Equal volumes of at least ten of these samples were mixed to obtain the pooled blank urine used in the preparation of calibration standard and quality control samples.

2.2. Chromatographic system

The chromatographic system is displayed in Fig. 2. Waters Model 510 pumps were used to deliver the mobile phases to both columns. The pumps delivering mobile phase to the first column were regulated by a Waters Model 680 system controller. Samples were injected by a Perkin-Elmer ISS-100 autosampler (Perkin-Elmer, Beaconsfield, UK) fitted with a 150-μl sample loop. Column-switching was achieved using two Knauer six port valves (Knauer, Berlin, Germany) connected to the system controller via relays. A sample loop (2 ml) was used to trap the eluate from the first column. The HPLC columns were kept at a constant temperature (40°C) within a Spark Holland HPLC column oven (Severn Analytical, Gloucester, UK). The eluate was monitored, at a wavelength of 240 nm, with a Model SM 4000 ultra violet absorbance detector (LDC Analytical, Stone, UK). The output from the detector was handled by a Hewlett-Packard 3350A laboratory automation system data collection system (Hewlett-Packard, Altrincham, UK).

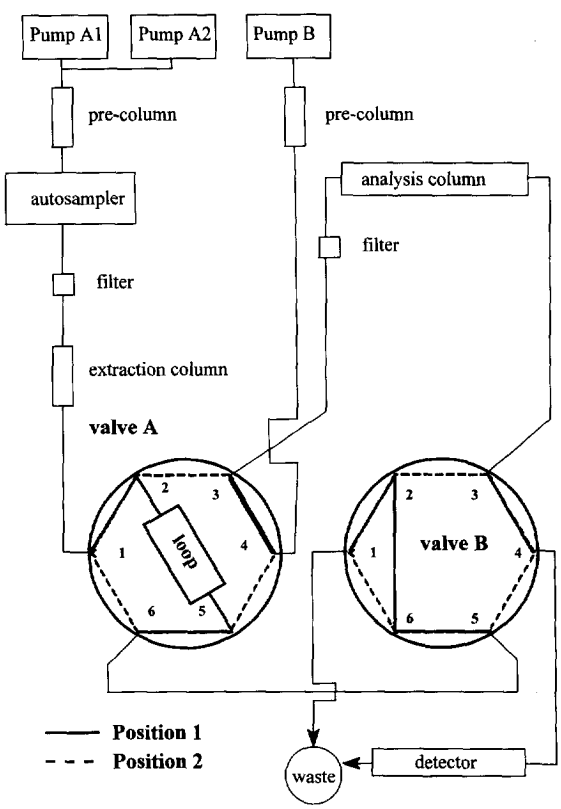


Fig. 2. Configuration of the HPLC system.

Silica pre-columns were not used because material emanating from them caused rapid build-up of back pressure in the HPLC system. Upchurch pre-column filters (0.5 μm, Fisons Scientific Equipment) were used to protect both the extraction and analytical columns. The extraction column (150×4.6 mm I.D., Hichrom, Reading, UK) was packed with 5 μm Hypersil 5-ODS. The analytical column (250×4.6 mm I.D., Fisons Scientific Equipment) was packed with 5 μm Zorbax TMS.

Polymethylpentene autosampler vials were obtained from HPLC Technology (Macclesfield, UK).

2.3. Mobile phases

Methanol–water (20:80, v/v) was used as the autosampler flush solvent. Mobile phase A, for the extraction column, was prepared in situ by the system controller, from methanol (pump A1, Fig. 2) and aqueous ammonium acetate (0.1%, w/v, pump A2, Fig. 2). Typically, a ratio of 28:72 (v/v) was needed, to obtain the desired retention time of 6–7 min on the first column for both analytes. Mobile phase B, for the analytical column (pump B, Fig. 2) was prepared by mixing acetonitrile and water, typically in the ratio 17:83 (v/v), in order to obtain retention times of 8.5–11 min for FPL 6635XX and 13–18 min for II. Both mobile phases were delivered at a flow-rate of 2 ml/min.

The mobile phases and flush solvent were sparged with helium before and during use.

2.4. Chromatographic procedure

Urine samples, if frozen, were allowed to reach ambient temperature, mixed and, if turbid, centrifuged. Portions of the samples were pipetted (500 μl) into autosampler vials.

At the start of each analysis batch the mean value of the separate retention times (t_A) of the two analytes on the extraction column was established. The HPLC system was set up (valve A at position 2, valve B at position 2 in Fig. 2) so that the 2-ml loop was out of line and the eluate from the extraction column passed directly to the detector. Successive injections (100 μl) of a calibration standard sample (2500 ng/ml) were performed until t_A was stable to within 0.02 min.

The sequence of events for the analysis of each sample is listed in Table 1. At the end of an analysis batch, the extraction column was flushed with methanol and then left with water–methanol (28:72, v/v) pumping through it at 0.1 ml/min. The analytical column was left with mobile phase B pumping through it at 0.1 ml/min.

2.5. Calibration and quality control of the assay

Stock solutions of I and II were prepared in methanol and added to pooled blank urine to obtain the calibration standard and quality control samples. These samples were stored, frozen, at or below −20°C. Calibration standard samples were prepared at concentrations of 10, 25, 50, 100, 250, 500, 1000, 2500 and 5000 ng/ml. Quality control samples were similarly prepared at concentrations of 25, 250 and 2500 ng/ml.

Calibration curves were constructed by the data system using peak height measurements from calibration standard samples. The calibration curves were fitted linearly, using a weighting factor for each standard, which was proportional to the reciprocal of the concentration squared. Pooled blank urine samples were also analysed in each batch, but not used in the calculation of the curves. Quality control samples were interspersed throughout each analysis batch to assess the acceptability of that batch.

2.6. Validation of the assay

The accuracy and precision of the method was investigated by the replicate analysis of samples containing the same concentrations of I and II as the calibration standard samples. Inter-assay performance was monitored using two samples at each concentration in each analysis batch. Intra-assay performance was monitored using six samples at each concentration in one analysis batch.

2.7. Specificity of the assay

The specificity of the method, with respect to interference from urine constituents, was evaluated by analysing urine samples from five male and five female volunteers not exposed to tipredane. These samples were analysed blank and spiked with the analytes at a concentration of 250 ng/ml.

Table 1
Chromatographic sequence for each analysis

Time after injection (min)	Valve positions (Fig. 2)		Event
	A	B	
0	1	1	Inject sample (100 μl)
t_A+0.6	2	1	Switch loop into path of mobile phase B and start collecting signal from detector
t_A+2.6	1	1	Switch loop back into line with mobile phase A
t_A+3.1→t_A+3.6	1	1	Increase methanol content of mobile phase A to 95%, v/v
t_A+3.6→t_A+5.6	1	1	Flush extraction column
t_A+5.6→t_A+6.1	1	1	Return mobile phase A to original composition
25	1	1	End of analysis

t_A=Retention time of the analytes on the extraction column.

Table 2
Capacity factors (k') determined of I and II on various reversed-phase columns[a]

Column	Length×I.D. (mm)	Manufacturer	Pore size (Å)	Carbon loading (%)	End-capped	k'			
						Acetonitrile–water (20:80, v/v)		Methanol–water (40:60, v/v)	
						I	II	I	II
Spherisorb CN	250×4.6	Phase Separations	80	3.5	No	NR	NR	NR	NR
Hypersil SAS (C_1)	125×4.6	Shandon	120	3	No	1.2	2.5	0.9	0.9
Zorbax TMS (C_2)	250×4.6	Dupont	70	5	No	2.5	3.8	1.7	1.7
Hypersil Phenyl	250×4.6	Shandon	120	5	No	2.5	2.5	1.4[b]	1.1
Microsorb C_8	250×4.6	Rainin	100	6	Yes	2.4	3.7	1.7	1.7
RAD-PAK Nova-Pak C_{18}	100×8	Waters	60	4	Yes	1.9	3.6	1.7	1.5
Resolve C_{18}	150×3.9	Waters	90	6	No	2.2	4.2	1.7	1.7
Spherisorb ODS (C_{18})	250×4.6	Phase Separations	80	7	Partial	3.0	3.9	2.1	1.5
Hypersil ODS (C_{18})	250×4.6	Shandon	120	10	Yes	1.3[b]	2.9	1.0	1.0
Zorbax ODS (C_{18})	250×4.6	Dupont	70	20	Yes	1.4[b]	3.3	1.2	1.2

NR= Not retained.
[a] Pore size, carbon loading and end-capping information was supplied by the manufacturers; all columns contain spherical particles 5 μm in diameter except for Waters Novapak C_{18} (4 μm).
[b] FPL 66365XX separates from its epimer, FPL 66364XX.

2.8. Limit of quantification

The limit of quantification for each analyte was based on an upper limit of 20% for precision and a range of 80 to 120% for accuracy.

2.9. Investigational work

The different columns which were evaluated for the extraction and analytical stages are listed in Table 2. Capacity factors were determined on a separate, single-column system, using a Hewlett-Packard HP 1090 chromatograph equipped with a diode array detector set at 40°C. The retention time of I in a mobile phase of 100% methanol was used as the hold-up time in the calculation of capacity factors.

3. Results and discussion

3.1. Chromatography

Chromatograms typical of calibration standard samples are displayed in Fig. 3. The linearity of the method was typified by the correlation coefficients (r) which varied in six analysis batches from 0.9965 to 0.9998 for I and 0.9966 to 0.9997 for II. The mean of the retention times for both analytes on the extraction column varied from 5.83 min in the first analysis batch to 6.30 min in the last analysis batch. The retention times on the analytical column ranged from 8.98 to 9.97 min for I and 13.91 min to 15.74 min for II in the first and last analysis batches, respectively. Neither the peak shape or the co-elution of the analytes on the extraction column deteriorated despite the injection of approximately 330 urine samples in these analysis batches.

3.2. Properties of the method

Intra- and inter-assay precision and accuracy data (Table 3) were acceptable between 25 and 5000 ng/ml. The intra-assay precision for I at 25 ng/ml of 25%, was considered acceptable as the inter-assay precision was 17.5% at this concentration. When blank samples from five male and five female volunteers not exposed to tipredane were analysed, neither I or II could be detected. When these samples were spiked at 250 ng/ml, the accuracy was 105% and 103% for I and II, respectively and the precision was 2.6% and 2.7% for I and II, respectively. These values were similar to those obtained for validation samples at the same.

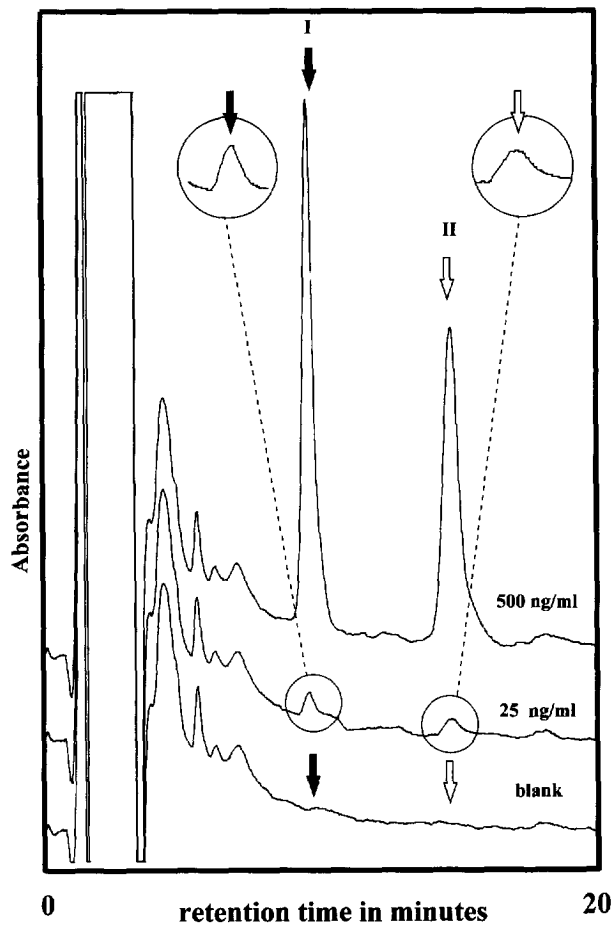


Fig. 3. Chromatograms obtained from human urine calibration standards.

Table 3
Intra-assay and inter-assay accuracy and precision for the determination of I and II in human urine

Spiked concentration (ng/ml)	Intra-assay ($n=6$)				Inter-assay ($n=12$)[a]			
	Accuracy (%)		C.V. (%)		Accuracy (%)		C.V. (%)	
	I	II	I	II	I	II	I	II
10	87.0	77.0	19.5	36.4	118	108	26.3	25.0
25	93.2	92.8	3.9	25.0	99.2	98.4	12.5	17.5
50	97.0	94.4	2.9	7.2	98.0	98.0	6.5	6.3
100	101	101	1.9	3.3	98.5	98.4	4.1	4.7
250	100	99.2	2.0	1.6	97.6	96.4	3.3	3.5
500	113	112	3.0	2.5	109	110	4.0	3.8
1000	107	106	3.2	1.9	102	102	3.8	3.7
2500	108	106	2.9	1.6	101	101	4.6	4.2
5000	104	102	3.8	2.1	98.0	98.4	4.7	3.9

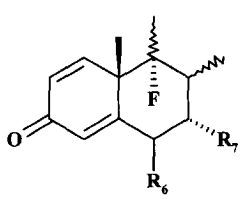
[a] Duplicate samples in six analysis batches.

The lower limit of quantification was therefore considered to be 25 ng/ml for both I and II. The highest concentration at which the properties of the method was investigated was 5000 ng/ml and this was therefore considered to be the upper limit of quantification of the method for both analytes.

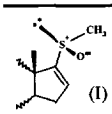
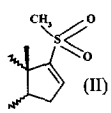
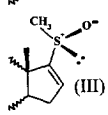
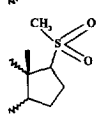
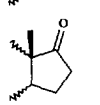
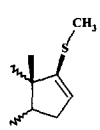
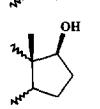
3.3. Specificity of the assay towards various other tipredane metabolites

As the human metabolism of tipredane is complex, the chromatographic behaviour of tipredane and a range of tentatively identified or putative metabolites on the extraction column was investigated (Fig. 4). All the des-hydroxy metabolites were strongly retained on the extraction column and did not elute within 10 min in a mobile phase of methanol–aqueous ammonium acetate (0.1%, w/v) (28:72, v/v). Both the 7α-hydroxy derivatives and all but one of the 6β-hydroxy derivatives eluted within 10 min. Only the epimer of I, FPL 66364XX (III, Fig. 4) eluted within 0.5 min of the analytes. The metabolites which were more strongly retained than the analytes on the extraction column were prevented from interfering with subsequent analyses by flushing the extraction column with 95% (v/v) methanol after the loop had been switched (Table 1).

When pooled urine samples spiked with these compounds at a concentration of 250 ng/ml were analysed by the method, no peaks were recorded at the retention time of II on the analytical column. However some metabolites gave small peaks corre-



AB rings

D-ring	R_6	R_7	Retention time in minutes
 (I)	OH	H	5.45
	H	H	>10†
 (II)	OH	H	5.55
	H	H	>10†
 (III)	OH	H	5.84
	OH	H	4.25
	H	OH	6.71
	OH	H	7.12
	H	OH	7.03
	H	H	>10†
	OH	H	>10†
	H	H	>10†
Tipredane	H	H	>10†

† Strongly retained, did not elute within ten minutes

Fig. 4. Retention times of various metabolites of tipredane on the extraction column.

sponding to I. As the retention times of these interferences on the extraction column greatly differed from that of I, the peak on the analytical column was assigned to the presence of this analyte as an impurity or degradation product. Only III eluted very close to I on both the extraction and analytical columns. However, this was not regarded as a serious problem as III is a minor human metabolite. Indeed, as supplied, I contained ca. 5% III, and the absorbance extinction coefficients were identical at 240 nm, it was thought preferable to quantify the epimers together.

3.4. Stability of I and II in human urine

Both analytes were found to be stable in human urine when stored at room temperature for two days, at 4°C for six days, or in a freezer at or below −20°C for three weeks, and when the urine samples were subjected to three freeze–thaw cycles. Under these storage conditions the recoveries for I were 98.8%, 101%, 94.0% and 87.5%, respectively at 25 ng/ml, 96.9%, 107%, 101% and 99.6%, respectively at 250 ng/ml and 96.9%, 101%, 93.2 and 97.2%, respectively at 2500 ng/ml. The corresponding recoveries for FPL 66366X were 107%, 108%, 102% and 91.3%, respectively at 25 ng/ml, 98.0%, 106%, 98.8% and 107% respectively, at 250 ng/ml and 97.6%, 102%, 92% and 102%, respectively at 2500 ng/ml.

3.5. Analysis of samples from a clinical study

This method was used to analyse urine samples from a clinical study where volunteers had been administered a dose of tipredane of 4 mg by a metered dose inhaler (4×1 mg). Representative

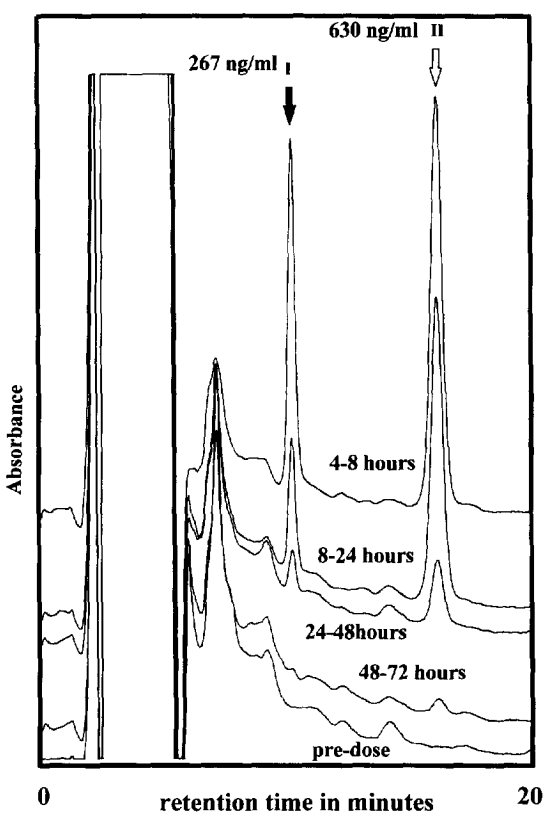
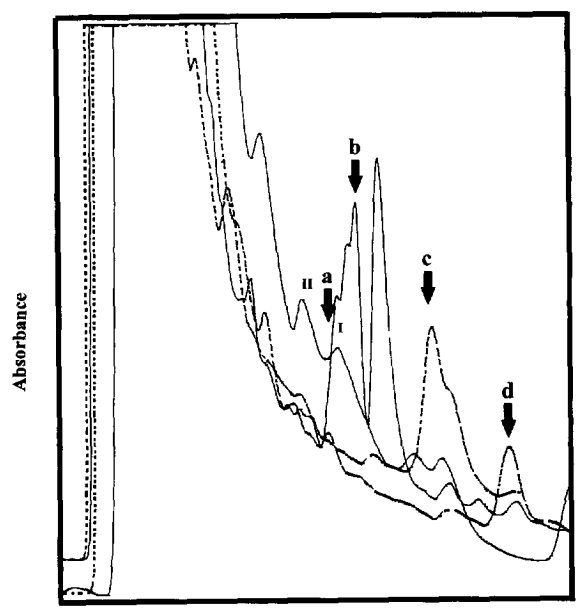


Fig. 5. Chromatograms of uine samples from a volunteer in a clinical study administered a dose of 4 mg of tipredane via a metered dose inhaler.



Chromatogram	Column	Dimensions (mm ID)	Injection Volume (µl)	Standard (ng/ml)
a	Spherisorb -CN	250 x 4.6	20	1000
b	Resolve ODS	150 x 3.9	100	1000
c	Hypersil SAS	125 x 4.6	100	1000
d	Hypersil ODS	150 x4.6	100	250

Fig. 6. Chromatograms of calibration standard samples obtained using various extraction columns. Mobile phases: acetonitrile–aqueous ammonium acetate (5:95, v/v) for (a) and methanol–aqueous ammonium acetate (0.1%, w/v) (25:75, v/v) for (b), (c), (d), all delivered at a flow-rate of 2 ml/min.

chromatograms are given in Fig. 5. Neither analyte was detected in any samples collected before the start of dosing. None of the samples taken after dosing resulted in concentrations above the top quality control sample, 2500 ng/ml. The analytes were still quantifiable in urine samples collected between 48 and 72 h after dosing.

3.6. Selecting the extraction column

Euerby et al. [8] reported that the selectivity for I and II of reversed-phase systems based on C_{18} columns was highly dependent on the nature of the organic constituent of the mobile phase. When an aqueous mobile phase containing methanol was used here, I and II co-eluted on several C_1, C_8 and C_{18} columns (Table 2). Only the stationary phases which weakly retained the analytes and were available as short columns of 125–150 mm in length were tested as extraction columns. Their performance was compared to that of Spherisorb CN, a -CN phase having been used as the extraction column in the determination of a different tipredane metabolite in rat urine [7]. As demonstrated by the chromatograms in Fig. 6, resolution of the analytes from the human urine background improved in the following order: 250 mm Spherisorb CN<150 mm Resolve ODS column (C_{18})<125 mm Hypersil SAS (C_1)<150 mm Hypersil ODS (C_{18}). The analytes eluted as one peak from the Hypersil ODS column, were partially separated on Hypersil SAS and Resolve ODS, and were completely resolved on Spherisorb CN. The chro-

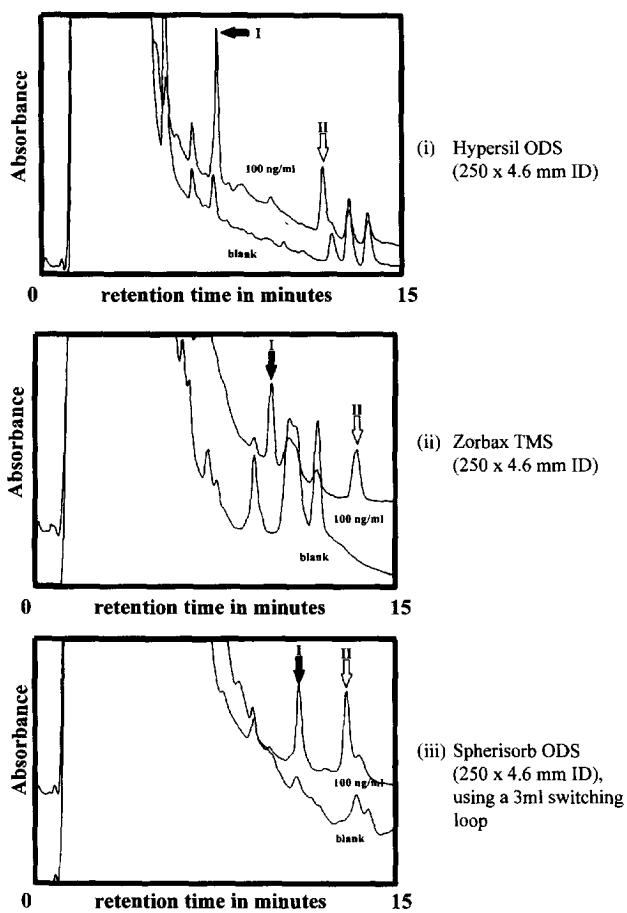


Fig. 7. Chromatograms from calibration standard and blank samples using Spherisorb-CN (250 mm×4.6 mm I.D.) as the extraction column and various analytical columns. Mobile phases: methanol–aqueous ammonium acetate (25:75, v/v) on the extraction column and acetonitrile–water (17.5:82.5, v/v) on the analytical column, both delivered at 2 ml/min.

matograms in Figs. 7 and 8, illustrating the use of Spherisorb CN and Resolve ODS as extraction columns, demonstrate that increasing the amount of the urine background collected in the switching loop, results in an increased level of both general and specific interferences on the analytical column. Consequently Hypersil ODS was selected as the extraction column.

3.7. Selecting the analytical column

Zorbax TMS was chosen for the analytical column because, as epitomised in Figs. 6–8, it was the best material for separating the analytes from each other and urine constituents, whatever extraction column was used. Switching 2 ml of aqueous methanol into the mobile phase containing aqueous acetonitrile has a small but significant effect, on the retention times on the analytical column. Hence a column containing Zorbax TMS (C_1) is more retentive for II than one of the same dimensions containing Spherisorb ODS (C_{18}) in the column-coupling system, despite having a smaller capacity factor in aqueous acetonitrile (Table 2).

3.8. Coupling the columns

To our knowledge the literature on column-switching assays emphasises the different selectivities of the extraction and analytical columns. The key to the performance of this assay is the major difference in the selectivities shown towards the two analytes by

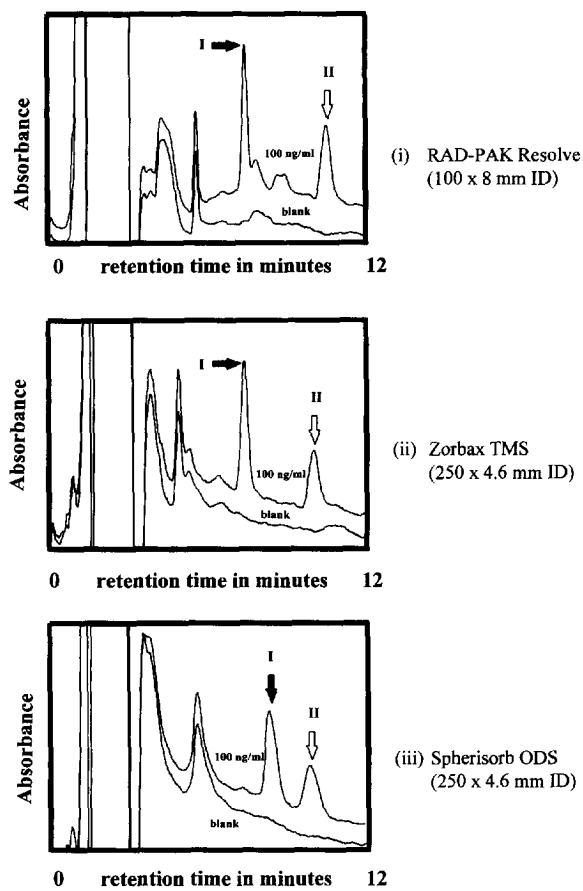


Fig. 8. Chromatograms from calibration standard and blank samples using Resolve ODS (150 mm×4.6 mm I.D.) as the extraction column and various analytical columns. Mobile phases: methanol–aqueous ammonium acetate (25:75, v/v) on the extraction column and acetonitrile–water (17.5:82.5, v/v) on the analytical column, both delivered at 2 ml/min.

the organic modifiers, methanol and acetonitrile, in the mobile phases used for extraction and analysis. These effects are so dominant that good chromatography can be surprisingly obtained if the same stationary phase, Resolve ODS, is used for both the analytical and extraction columns, as illustrated in Fig. 8(i). These effects are also responsible for the unusual nature of the finalised system in that a non-polar C_{18} column is used for the extraction and a more polar C_1 column used for the analysis.

4. Conclusion

An automated column-switching HPLC assay, with a lower limit of quantification of 25 ng/ml, has been developed for the major human metabolites of tipredane, I and II, in human urine. Urine samples are analysed directly and the method is robust, accurate, precise, selective and sensitive. Consequently it has been successfully used to analyse samples from a clinical study.

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References

[1] R.G. Devlin, A.A. Sugerman, K.J. Kripalani, A.V. Dean and F.C. Bocobo, Semin. Derm., 6 (1987) 23.
[2] S.J. Lan, J. Mitroka, S.H. Weinstein, B.N. Lutsky, C.A. Free, R.J. Wojnar, R.C. Millonig and B.H. Migdal, J. Steroid Biochem., 31 (1988) 825.
[3] S.J. Lan, L.M. Scanlan, R.K. Varma, B.M. Warrack, S.E. Unger, M.A. Porubcan and B.H. Migdalof, Drug. Metab. Dispos., 17 (1989) 532.
[4] K.J. Kripalani and J.G. Mitroka, in E. Christopher and H. Maibach (Editors), Topical Therapy with Corticosteroids, New Advances, Proceedings of a Symposium held in Conjunction with the 17th World Congress of Dermatology, May 28, 1987, Berlin, Germany pp. 94– 101. Excerpta Medica, Princeton, NJ.
[5] M.A.J. Bayliss, J.R. Preston, D. Wilkinson, D.J. Wilkinson and W.J.S. Lockley, J. Pharm. Biomed. Anal., in press.
[6] M.A.J. Bayliss, J.R. Preston, C.M. Gilbert, V.J.C. Willson and D. Wilkinson, manuscript in preparation.
[7] M.A.J. Bayliss, P.R. Baker and D. Wilkinson, J. Chromatogr. B, submitted for publication.
[8] M.R. Euerby, C.M. Johnson and S.C. Nichols, Anal. Proc., 30 (1993) 46.