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Determination of a major metabolite of tipredane in rat urine by high-performance liquid chromatography with column switching

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Abstract

An automated method, based on column-switching reversed-phase high-performance liquid chromatography, has been developed for the determination of a major metabolite of tipredane in rat urine. Samples are injected directly onto a cyanopropyl extraction column. The portion of eluate containing the metabolite is switched, via an injection loop, onto an octadecylsilane analytical column. The limit of quantification of the method was 25 ng/ml for a 20 μl injection volume of urine. The intra-assay precision (0.7–4.8%) and accuracy (94–105%), and the inter-assay precision (2.7–12.6%) and accuracy (94–105%), were acceptable. The analyte was found to be stable in rat urine when stored at room temperature for six days, in a freezer at or below −20°C for twelve weeks, and when the samples were subjected to two freeze–thaw cycles. No significant interference was observed from tipredane and its major human metabolites, or urine constituents in male and female rats. The method was successfully used to analyse samples from a long-term toxicology study.

Keywords: Tipredane

1. Introduction

Tipredane ((11β-17α-(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.

During the safety evaluation programme for tipredane, monitoring of exposure to the drug in the rat was required during long-term inhalation toxicology studies. After administration by inhalation, the drug undergoes rapid and complex metabolism [2,3]. When rats in metabolism studies were dosed with ^{3}H-labelled tipredane, the parent compound could not be detected in either urine or plasma [4]. The major urinary metabolite in the male rat, FPL 67336XX (I, Fig. 1), was therefore used to estimate exposure in both the male and female rat.

High-performance liquid chromatography (HPLC) with column switching, as evident from recent reviews [5–11], has become a popular means of automating the extraction and analysis of biological fluids. This technique has been used to analyse

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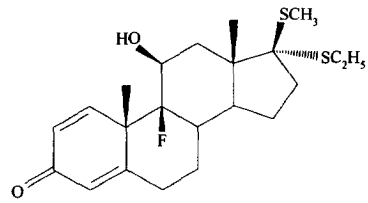
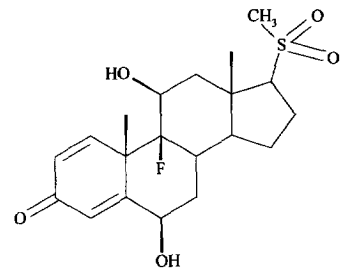


Tipredane



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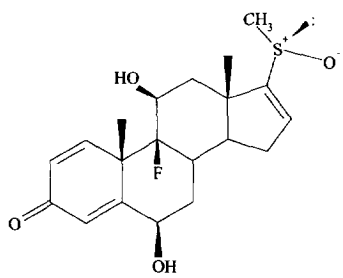

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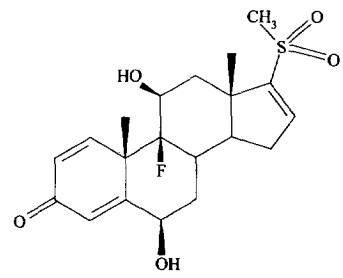

IV

Fig. 1. Structures of tipredane, FPL 67336XX (I), FPL 66365XX (II) and FPL 66366XX (III).

another anti-inflammatory drug, nedocromil sodium, in human urine [12]. Column-switching, in the heart cutting and loop transfer mode [13], was explored with regard to the determination of I in rat urine, as alternative HPLC methodology did not have the required sensitivity and specificity.

Urine samples were directly injected into the HPLC system, after minimal pre-treatment, thus avoiding any manual sample extraction. Two reversed-phase HPLC columns were used in a heart cutting column-switching configuration. The initial separation was performed on a short, moderately non-polar, cyanopropylsilane column. The portion of the column eluate from the first column containing the analyte was switched, via an injection loop, to a second, non-polar, octadecylsilane column, where the final separation was carried out.

The properties of the assay and its use in support of a toxicology study are described here.

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).

The HPLC reagents were of HPLC grade and were purchased from Fisons Scientific Equipment (Loughborough, UK). Water was purified by distillation and passage through a Waters Milli-Q Plus Water Purifi-

cation System supplied by Millipore (Waters Chromatography Division, Harrow, UK).

Blank rat urine samples were obtained from rats not exposed to tipredane and were stored at or below −20°C. Equal volumes of at least ten of the blank urine samples were mixed to obtain the pooled blank urine used for the preparation of calibration standard and quality control samples

2.2. Chromatographic system

The chromatographic system is shown in Fig. 2. Waters Model 510 pumps were used to deliver the mobile phases to both columns. The pump delivering mobile phase to the first column was regulated by a Waters Model 680 system controller. Samples were injected by a Perkin-Elmer ISS-100 autosampler (Perkin-Elmer, Beaconsfield, UK) that was 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

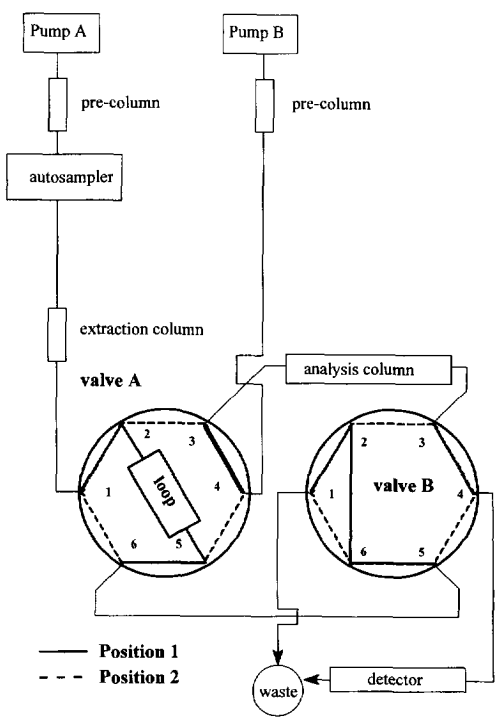

Fig. 2. Configuration of the HPLC system.

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 242 nm, with a Model SM 4000 ultraviolet 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).

A pre-column (250×4.6 mm I.D.; Hichrom, Reading, UK) packed with silica (40 μm) was positioned after each pump to saturate the mobile phases with silica and to prevent impurities from collecting on the extraction column. The extraction and analytical columns (both 250×4.6 mm I.D.; Fisons Scientific Equipment) were packed with Hypersil 5-CPS and Hypersil 5-ODS, respectively.

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

The mobile phase for the extraction column (Pump A, Fig. 2) was prepared by mixing methanol with aqueous ammonium acetate (0.1%, w/v) (20:80, v/v). The mobile phase for the analytical column (Pump B, Fig. 2) was prepared by mixing acetonitrile, methanol and water (8.8:18.8:72.5, v/v). The mobile phases were delivered from pumps A and B at flow-rates of 2 and 1 ml/min, respectively.

2.4. Chromatographic procedure

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

At the start of each analysis batch, the retention time (t_A) of I on the extraction column was established. The HPLC system was configured (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 (20 μl) of the top calibration

standard sample (10 000 ng/ml) were performed until t_A was stable to within 0.02 min for successive injections.

The sequence of events for the analysis of each sample is listed in Table 1. At the end of an analysis batch, both columns were left with the flush solvent pumping through them at a flow-rate of 0.1 ml/min.

2.5. Calibration and quality control of the assay

Stock solutions of I 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 by spiking pooled urine with I at concentrations of 25, 50, 100, 200, 500, 1000, 2500, 5000 and 10 000 ng/ml. Quality control samples were prepared similarly, at concentrations of 50, 500 and 8000 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 were 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 were investigated by the replicate analysis of samples containing the same concentrations of I 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 four male and four female rats that had not been exposed to tipredane. These samples were analysed blank and spiked at a concentration of 500 ng/ml.

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.

3. Results and discussion

3.1. Chromatography

Chromatograms from typical calibration standard samples are displayed in Fig. 3. The linearity of the method was characterised by the correlation coefficients (r), which varied from 0.9978 to 0.9997 in seven analysis batches. The retention times on the

Table 1
Chromatographic sequence for each analysis

Time after injection (min)	Valve positions (Fig. 1)		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+4.6	1	1	Switch loop back into line with mobile phase A
20	1	1	End of analysis

t_A=The retention time of II on the extraction column.

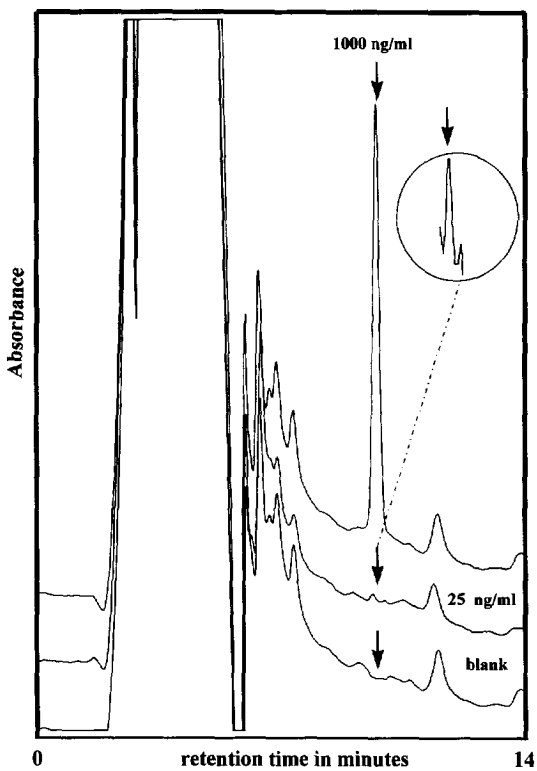

Fig. 3. Chromatograms obtained from rat urine calibration standards.

extraction column and analysis columns varied from 5.85 and 9.82 min, in the first analysis batch, to 5.61 and 9.64 min, in the last analysis batch. There was no marked deterioration in the peak shape on the extraction column despite the injection of approximately 300 urine samples.

3.2. Properties of the method

Intra- and inter-assay precision and accuracy (Table 2) were acceptable between 25 and 10 000 ng/ml. No significant interferences were seen in the blank samples from four male and four female rats that had not been exposed to tipredane, the mean response being 10.0±6.7 ng/ml. When these samples were spiked at 500 ng/ml, the accuracy was 103% and the precision was 2.8%. These values were similar to those obtained for validation samples at the same concentration. Neither tipredane or its major human metabolites [14], FPL 66365XX (II, Fig. 1) and FPL 66366XX (III, Fig. 1), interfered with the method.

The lower limit of quantification was therefore considered to be 25 ng/ml. The highest concentration at which the properties of the method was investigated was 10 000 ng/ml and this was therefore the upper limit of quantification of the method.

3.3. Stability of I in rat urine

The stability of I in pooled rat urine was investigated using quality control samples. No degradation of I was detected after the samples were stored at room temperature for six days, or in a freezer at or below −20°C for twelve weeks, or when the samples were subjected to two freeze–thaw cycles. Under these storage conditions, the recoveries were 93.2, 104 and 93.9%, respectively, at 50 $ng \cdot ml^{-1}$, 102, 97.9 and 101%, respectively, at 500 $ng \cdot ml^{-1}$, and 102, 96.6 and 101%, respectively, at 800 $ng \cdot ml^{-1}$.

Table 2
Intra-assay and inter-assay accuracy and precision for the determination of II in rat urine

Spiked concentration (ng/ml)	Intra-assay ($n=6$)		Inter-assay ($n=12$)[a]	
	Accuracy (%)	Precision C.V. (%)	Accuracy (%)	Precision C.V. (%)
25	99	4.8	96	12.6
50	96	1.4	94	4.6
100	94	1.4	94	3.2
200	97	1.7	100	3.6
500	95	1.0	96	3.3
1000	98	0.70	99	3.9
2500	105	0.94	105	2.9
5000	97	0.84	98	2.7
10 000	99	1.3	100	5.5

[a] Duplicate samples in six analysis batches.

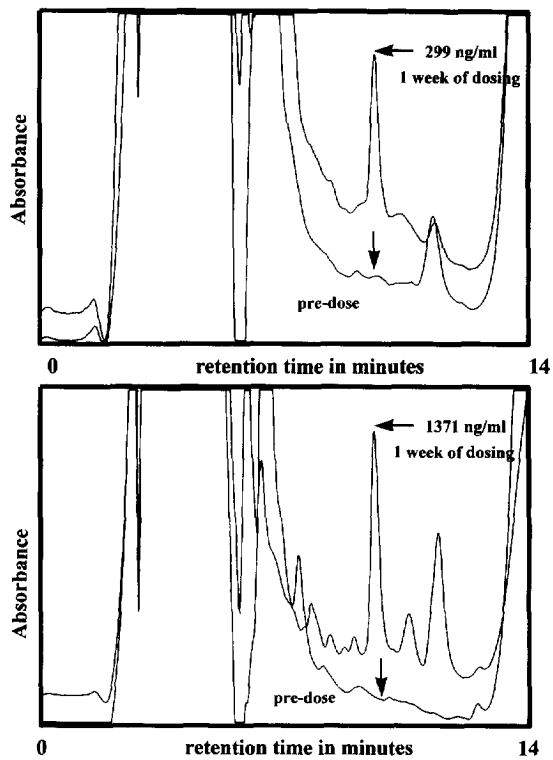

Fig. 4. Chromatograms from rat urine samples collected during an inhalation toxicology study. (Top) Male rat receiving a dose of 0.08 mg/kg/day. (Bottom) Female rat receiving a dose of 2 mg/kg/day.

3.4. Analysis of samples from a toxicology study

This method was used to support a long-term toxicology study in the rat. I was not detected in any samples collected from the rats before the start of dosing, or in samples taken from the control group up to 52 weeks after the start of dosing. Analysis of samples from the rats receiving doses of tipredane gave results between the limit of quantification, 25 ng/ml, and the concentration of the top quality control sample, 8000 ng/ml, in all cases. Representative chromatograms from rats receiving doses of 0.08 mg/kg/day and 2 mg/kg/day are given in Fig. 4.

4. Conclusion

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

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