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Bioanalysis of *p*-trifluoromethylmandelic acid and Mosher's acid by chiral gas chromatography and fluorine nuclear magnetic resonance to study chiral inversion: application to rat urine samples

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Abstract

Methods for the nuclear magnetic resonance and gas chromatographic analysis of the enantiomers of *p*-trifluoromethylmandelic acid (*p*-TFM) and Mosher's acid (α-methoxy-α-(trifluoromethyl)phenylacetic acid) present in rat urine samples are described. Gas chromartography was performed using cyclodextrin capillary columns with both compounds analysed following derivatisation with methanolic HCl. Nuclear magnetic resonance was performed directly on the untreated urine samples following addition of beta-cyclodextrin. The methods were suitable for the determination of the individual enantiomers of the analytes in urine. Analysis of the rat urine samples indicated that the *p*-TFM had undergone a unidirectional enantiomeric interconversion in vivo, while the enantiomers of Mosher's acid were excreted unchanged.

Keywords: Enantiomer separation; *p*-Trifluoromethylmandelic acid; Mosher's acid

1. Introduction

Improved understanding of the role of chirality in the biological action of drugs has led to many pharmaceutical companies developing only the pharmacologically active single enantiomer [1,2]. For some drugs however there is the possibility of interconversion from one enantiomer to the other in vivo, as has been demonstrated for thalidomide and ibuprofen etc. [3,4]. Given the possibility of chiral inversion regulatory authorities are increasingly asking for evidence that this does not occur following the administration of single enantiomers. The demonstration of metabolic chiral inversion would obviously have profound consequences for the development of a new drug in terms of the increased toxicology and bioanalytical workload that would result. A better understanding of the factors facilitating such interconversions would thus greatly aid drug discovery by enabling this unwanted feature to be designed out of candidates at an early stage. It has been suggested that in the case of profens there is a requirement for abstraction of a single proton from the α-methine position for chiral inversion to occur [5].

Here the in vivo chiral inversion of *p*-trifluoromethylmandelic acid (*p*-TFM) and the closely related compound Mosher's acid (see insets to Figs. 1

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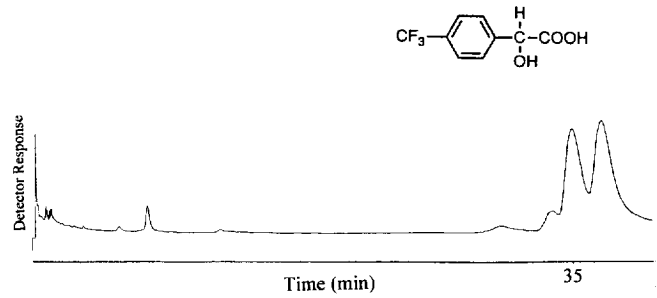

Fig. 1. Chromatogram of *rac-p*-TFM (0.80 mg/ml) following derivatisation with methanolic hydrochloric acid. Conditions: B-PH column, helium carrier gas at 45 kPa, oven temperature 80°C, split injection (split flow 50 ml/min) of 1 μl, ECD (nitrogen make-up at 30 ml/min).

and 2 for structures) have been investigated in the rat. *p*-TFM contains a hydrogen on the chiral centre, and would thus be expected to be subject to chiral inversion (as suggested by preliminary studies [6]), whereas Mosher's acid does not. Both compounds were dosed to rats and urine collected for 48 h. Following the failure of attempts to develop chiral high-performance liquid chromatographic (HPLC) separations for the analytes capillary gas chromatographic (GC) methods to determine the enantiomeric ratios in urine were developed and validated. The presence of trifluoromethyl groups in both analytes also enabled ^{19}F-nuclear magnetic resonance (NMR) to be used to analyse the urine samples. This provided an alternative method to study the metabolism of these compounds, and gave an independent means of validating the results provided by GC.

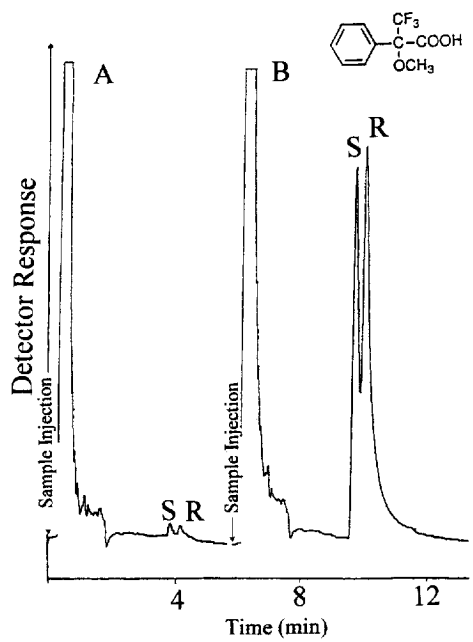

Fig. 2. Chromatograms of Mosher's acid following derivatisation with methanolic hydrochloric acid. (A) 0.033 mg/ml *S*-Mosher's acid, 0.030 mg/ml *R*-Mosher's acid, (B) 1.309 mg/ml *S*-Mosher's acid, 1.209 mg/ml *R*-Mosher's acid. Conditions: G-TA column, helium carrier gas at 45 kPa, oven temperature 100°C, split injection (split flow 50 ml/min) of 1 μl, ECD (nitrogen make-up at 30 ml/min).

2. Experimental

2.1. Chemicals

rac-p-TFM and *R*-(+)- and *S*-(−)-Mosher's acid were obtained from Sigma–Aldrich (Poole, UK). Analytical reagent-grade hexane and hydrochloric acid and HPLC-grade methanol, were obtained from Fisons (Loughborough, UK).

2.2. Instrumentation

2.2.1. Gas chromatography

A Hewlett-Packard 5890A GC with an electron-capture detector (ECD) was used with a HP3396A integrator and HP7673A automatic sampler. The capillary columns used were Chiraldex B-PH (10 m×0.2 mm I.D.) for *p*-TFM and Chiraldex G-TA (10 m×0.2 mm I.D.) for Mosher's acid (both columns from Astec, Whippany, NJ, USA). Gases were obtained from BOC (Guildford, UK). Split injection (flow-rate 50 ml/min) was used throughout.

2.2.2. NMR

NMR spectroscopy was performed on a Bruker AC250 NMR spectrometer, operating at 235.36 MHz for fluorine nuclei. ^{19}F NMR spectra were acquired with CPD decoupling to remove all ^{1}H coupling interactions, using a 90° pulse width with relaxation delay 2.0 s. Samples were recorded with varying numbers of scans depending on signal-to-noise requirements, collected into 32 768 data points.

2.3. Dosing protocol

All rat dosing and urine collection was performed at the University of Surrey. For *p*-TFM, two male rats were dosed with *rac-p*-TFM (100 mg/kg), for Mosher's acid three male rats were dosed (50 mg/kg) with *R*-, *S*- and (*R,S*, 50:50), respectively. Animals were dosed intravenously with the test compounds administered in sodium bicarbonate/isotonic saline. All rats were 250(±10) g, and urine collection was performed at 0–24 h and 24–48 h. Samples were stored frozen until analysis. Prior to the GC analysis of the rat urine samples, the methods were validated, with precision evaluated on a day-to-day and within-day basis, using spiked samples prepared in pooled human urine.

2.4. Sample preparation for gas chromatography

Both compounds were chromatographed as methyl esters by reaction with methanolic hydrochloric acid. 2 *M* hydrochloric acid (HCl) in methanol (2 ml) was added to 200 μl of urine and the solution heated to 100°. For *p*-TFM solutions were left to react for 60 min, for Mosher's acid the reaction time was 2 h. These times were determined by comparing derivative yields from a range of reaction times. After reaction the solutions were allowed to cool (30 min) and then hexane (400 μl) was added and the solutions centrifuged for 5 min at 500 *g*. 1 μl of the hexane layer was then injected into the GC system.

For method validation stock solutions of *R,S*-Mosher's acid (prepared from the single enantiomers) and *rac-p*-TFM were prepared in pooled human urine and used to prepare calibration standards and spiked solutions at three concentrations. Fresh calibration curves were prepared on each day of analysis and used to determine the concentrations of the spiked solutions. The rat urine samples were analysed along with the validation samples on the final day.

2.5. Sample preparation and method development for NMR

Samples of urine (500 μl) were taken and made up to 600 μl from a stock solution of D_2O (100 μl), which acted as a field frequency lock for the NMR experiments. A small quantity of trifluorethanol was contained in this stock solution as a chemical shift reference set to −77.02 ppm. For the determination of the enantiomeric composition of the samples a suitable method was developed in D_2O solution. The best chiral solvating agent (CSA) was found to be beta-cyclodextrin which caused doubling of the CF_3 signals in both compounds. This method was applied to the samples as follows. Firstly the metabolites present in the urine were quantified using an internal standard (TFM for Mosher's acid, Mosher's acid for TFM). Typically 500 μl of urine was added to 500 ul of the appropriate standard solution containing 500 μg/ml reference standard. Enantiomeric composition was then determined by addition of beta-cyclodextrin (Sigma–Aldrich, cell culture grade) at a ratio 1:1 mole equivalent CSA/metabolite.

3. Results and discussion

Currently most chiral bioanalysis is carried out using HPLC methods. However, for the two compounds investigated here satisfactory separations on α-acid glycoprotein (AGP) and both native and acetylated β-cyclodextrin columns were not readily obtained (unpublished results). A range of cyclodextrin chiral stationary phases are now available for GC and these were investigated [7,8].Though the technique is limited to volatile compounds it was found in this study that a simple derivatisation with methanolic HCl, directly on the urine samples, gave derivatives suitable for GC. As both compounds possess an organohalogen functional group the highly selective ECD was used.

The methyl esters of the *p*-TFM enantiomers were unresolved on the G-TA column, but were separated on the B-PH column. The opposite was observed

Table 1
Resolution data for *p*-TFM and Mosher's acid

Compound	Column	k_1'	k_2'	α	First eluted enantiomer
p-TFM	B-PH	163.5	172.7	1.056	[a]
Mosher's acid	G-TA	13.6	14.7	1.084	*S*

[a] Enantiomer elution order unknown.

with the methyl esters of the enantiomers of Mosher's acid, which were not resolved on the B-PH column but were separated with the G-TA. Table 1 lists the resolution data for both compounds. Chiral separation was confirmed with the single enantiomers in the case of Mosher's acid, with the enantiomeric elution order on the G-TA column being *S*-(−) followed by *R*-(+). The single enantiomers of *p*-TFM were unavailable. The 2 peaks obtained with *rac*-*p*-TFM on the B-PH column were assumed to be due to the individual enantiomers. Separations were optimised with pure standards before attempting analysis from urine. The direct treatment of urine samples with methanolic HCl was found to produce the required methyl esters quite readily. Example chromatograms of the separations achieved for both racemates when present in urine samples are shown in Figs. 1 and 2.

The method was evaluated by spiking known concentrations of racemic *p*-TFM or Mosher's acid into control urine and assaying these samples on five or six days, along with study samples. The calibration curves ($y=mx+c$) were linear ($r\geq 0.995$) over the ranges, 0.39 mg/ml to 7.95 mg/ml for *rac*-*p*-TFM and 0.03 mg/ml to 1.21 mg/ml for *R*-(+)- and 0.03 to 1.31 mg/ml for *S*-(−)-Mosher's acid. The slope of the calibration curve for *p*-TFM had a C.V. of ±11.4% and ±13.5% ($n=5$) for the first and second eluting enantiomer respectively, and

Table 3
Method validation for *p*-TFM in urine ($n=5$)

Enantiomer	Concentration spiked (mg/ml)	Within-day C.V. (%)	Day-to-day C.V. (%)
First eluting	0.80	6.3	7.8
	1.99	4.9	6.9
	3.18	2.9	7.6
Second eluting	0.80	5.8	7.0
	1.99	4.7	7.3
	3.18	3.3	7.8

±20% for both enantiomers of Mosher's acid. The limit of reliable quantitation was 0.33 mg/ml for the *p*-TFM enantiomers and 0.12 mg/ml and 0.14 mg/ml for *R*-(+)- and *S*-(−)- Mosher's acid, respectively. These were determined based on the method of Bonate [9] modified by Miller and Miller [10], using the intercept of the calibration curve. The precision of both methods was acceptable both day-to-day and within-day, (Table 2 for Mosher's acid and Table 3 for *p*-TFM).

Both methods were thus suitable for the determination of the enantiomer levels in the rat urine samples. The use of split injection can result in variations in the actual amount injected on column due to fluctuations in split vent flow and so it is anticipated that use of an injection standard to negate these variations would further improve the methods if required. Furthermore use of a suitable internal standard would allow for any sample loss during sample preparation.

The enantiomer concentrations found are shown along with details of dosing in Table 4. The rats dosed with Mosher's acid excreted the dosed enantiomer(s) unchanged.

This result was confirmed by analysis of the same

Table 2
Method validation for Mosher's acid in urine ($n=6$)

Enantiomer	Concentration spiked (mg/ml)	Within-day C.V. (%)	Day-to-day C.V. (%)
R	0.065	10.4	22
R	0.52	8.3	5.2
R	1.09	4.7	3.6
S	0.065	12.2	25
S	0.52	8.5	5.1
S	1.09	4.5	3.7

Table 4
Rat dosing and enantiomer concentrations

Rat No.	Compound dosed	Sample time (h)	Urine volume (ml)	Concentration of 1st eluting enantiomer (mg/ml)	Concentration of 2nd eluting enantiomer (mg/ml)	Total excreted (mg)	% of dose
1	*rac-p*-TFM	0–24	3.5	2.99	0	10.4	41.6
1		24–48	5.5	0	0		
2	*rac-p*-TFM	0–24	7.5	1.83	0	13.7	54.4
2		24–48	9.5	0	0		
3	*R*-Mosher's acid	0–24	7.0	0	1.74	12.2	48.7
3		24–48	7.0	0	0.07	0.49	1.96
4	*S*-Mosher's acid	0–24	11.5	0.92	0	10.6	42.3
4		24–48	11.0	0	0		
5	*R,S*-Mosher's acid	0–24	10.5	0.69	0.71	14.7	58.8
5		24–48	12.0	0	0		

samples using ^{19}F NMR following the addition of beta-cyclodextrin to the samples (Fig. 3A–C).

Excretion of the administered Mosher's acid via the urine was rapid, with the majority, approximately 50% of the dose administered, recovered in the 0 to 24 h urine collection and only trace amounts detectable in the 24 to 48 h samples. The fate of the remainder of the dose remains unknown, but was presumably excreted via the bile.

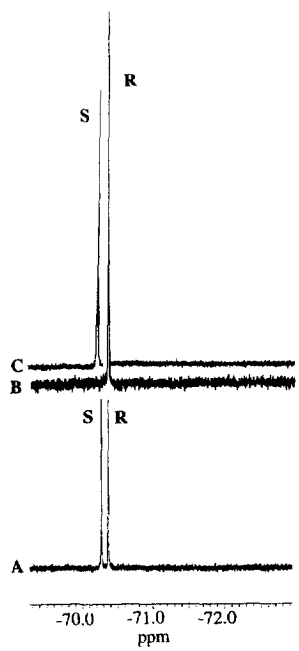

Fig. 3. ^{19}F NMR of urine samples, following the addition of beta-cyclodextrin, from rats administered (A) racemic (B) *R*- and (C) *S*-Mosher's acid. In all cases the administered enantiomer was the only one detected in the samples.

Rats dosed with *rac-p*-TFM, showed the presence of only one enantiomer in the samples when analysed by GC, confirming preliminary results obtained previously using ^{19}F NMR [6]. This result was further supported by the examination of these urine samples by ^{19}F NMR which showed the presence of one major and two more minor peaks. The signals corresponded to the *p*-TFM itself and putative ester glucuronide and *p*-TFM-glyoxylic acid metabolites. Following the addition of beta-cyclodextrin to the sample no separation of the resonance for *p*-TFM into separate signals corresponding to the individual enantiomers was seen, indicating that only one was present in the sample (Fig. 4) and see also [6]. This result was thus consistent with that produced by GC analysis, and similar to that seen in previous work [6].

The presence of a single enantiomer of *p*-TFM in the samples could have been the result of extensive metabolism, selective uptake and storage of the undetected enantiomer into tissues, or conversion of the undetected enantiomer into its antipode. The inversion of the undetected enantiomer is the most likely explanation as the concentration of the detected enantiomer is high (and other metabolites are also present, see earlier), moreover this type of

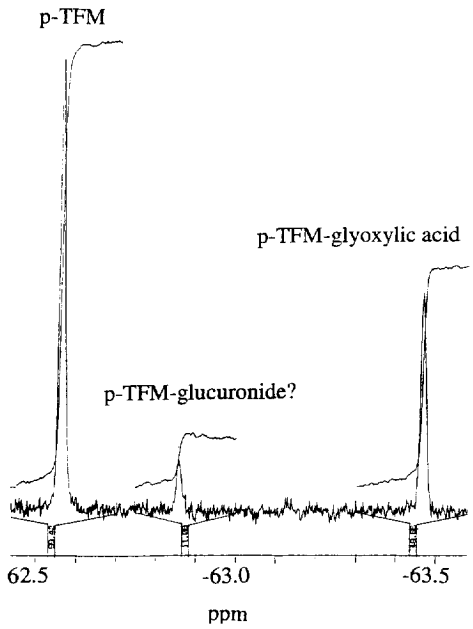

Fig. 4. ^{19}F NMR of rat urine, following addition of beta-cyclodextrin, from a rat administered *p*-TFM. Only one enantiomer was present in these samples.

metabolic inversion has been observed for mandelic acid [11], and for the structurally related profens [3–5]. Interconversion of the enantiomers during sample preparation can be discounted as this was not seen with standards.

As with Mosher's acid renal excretion was rapid and the kidney represented a major route of elimination for *p*-TFM. Thus about 50% of the dose was excreted as *p*-TFM, and additional material (unquantified) was also present as metabolites. The remainder of the dose was presumably excreted in the faeces.

In the absence of authentic standards we have not been able to easily determine which enantiomer of *p*-TFM is present in the urine samples from this study. In the case of ibuprofen, in vivo the inactive *R*-enantiomer is converted into the active *S*-enantiomer. The *R*-ibuprofen is converted to a thioester via acylcoenzyme A synthetase (CoA). The thioester subsequently undergoes racemisation to give the *S*-ibuprofen-CoA thioester, which is then hydrolysed to yield the *S*-ibuprofen. This interconversion is unidirectional as *S*-ibuprofen does not form the CoA-thioester. For mandelic acid the direction of chiral inversion proceeds, via the same mechanism, from *S* to *R* [11]. The stereoselective step in the mechanism is the formation of the CoA-thioester, which requires abstraction of a proton at the α-methine position. The difference in the fates of *p*-TFM and Mosher's acid is undoubtedly due to the presence of the alpha hydrogen on the chiral centre of the former, enabling metabolic inversion to take place. This also accounts for the lack of interconversion with the enantiomers of Mosher's acid, as lacking an α-hydrogen, it is incapable of forming the required CoA-thioester. In the absence of authentic standards of *R* and *S p*-TFM it is not possible, using these methods, to establish which enantiomer is being inverted.

4. Conclusion

The usefulness of capillary GC for bioanalysis, and of chiral GC for the determination of metabolic inversion, was confirmed with the methods developed here for *p*-TFM and Mosher's acid. The sample preparation required for the analysis of the urine samples was rapid and simple, and combined with the high specificity of the ECD allowed for chromatograms relatively clear of interferences. The detection of only one enantiomer of *p*-TFM in the rat urine samples suggests that *p*-TFM undergoes an enantiomeric interconversion in vivo similar to that observed with the profen non-steroidal anti-inflammatory drugs. ^{19}F NMR proved useful as a means of providing independent confirmation of the results obtained by GC with regard to enantiomeric composition of the analytes, and also enabled metabolite profiles to be obtained.

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