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Determination of the geometrical diarylpropenamine isomers in feces by high-performance liquid chromatography

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Abstract

Diarylpropenamine derivatives are a class of compounds which have been evaluated as potential drug candidates. Here a specific and reproducible HPLC method for the determination of *cis*- and *trans*-isomers of the unsubstituted derivative, 3-(4′-bromo-[1,1′-biphenyl]-4-yl)-3-(4-*X*-phenyl-*N*,*N*-dimethyl-2-propen-1-amine (I, where *X*=H) in feces is described. The analyte I and internal standard, nitro derivative (II, where *X*=NO_2), were isolated from the basified biological matrix using a liquid–liquid extraction with ethyl acetate followed by a solid-phase procedure performed on a silica cartridge. The organic phase was evaporated to dryness, the residue was reconstituted in mobile phase and injected into the HPLC system. The analytes were eluted with ethyl acetate–hexane–triethylamine (59:40:1) in HPLC column (silica) and detected by UV spectrophotometry at 272 nm. Linearity, precision and accuracy data for feces standards after extraction were acceptable. The method has been applied to analyses of feces samples from rats dosed with I, in which it could be anticipated that fecal excretion is quantitatively the major route for I elimination. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Geometrical isomers; Diarylpropenamine

1. Introduction

Diarylpropenamine derivatives are a promising class of compounds which have previously been examined as potential analgesic, antihistaminic, antispasmodic, analeptic, antidepressant and anti-infective agents [1–6]. Particularly, 3-(4′-bromo-[1,1′-biphenyl]-4-yl)-3-(4-*X*-phenyl)-*N*,*N*-dimethyl-2-propen-1-amine (where *X*=H, halogens, CH_3, OCH_3, NO_2 or OH, see Fig. 1), have been proved to be highly active against *Trypanosoma cruzi* infections, the causative agent of Chagas' disease [7,8]. In addition, they combined in vitro high activity against the three life-cycle stages of the parasite and low levels of cytotoxicity to mammalian cells and of bacterial acute toxicity in *E. coli* [9,10].

Chemically, these diarylpropenamines are characterized by para-substitutions on the phenyl moiety (Fig. 1), from which eight derivatives have been synthesized in our laboratory [9], with high purity and good yields (50–62%). Due to the presence of a double bond at the carbon 2, they are obtained as an isomeric mixture of *cis*–*trans* (1:1). For practical

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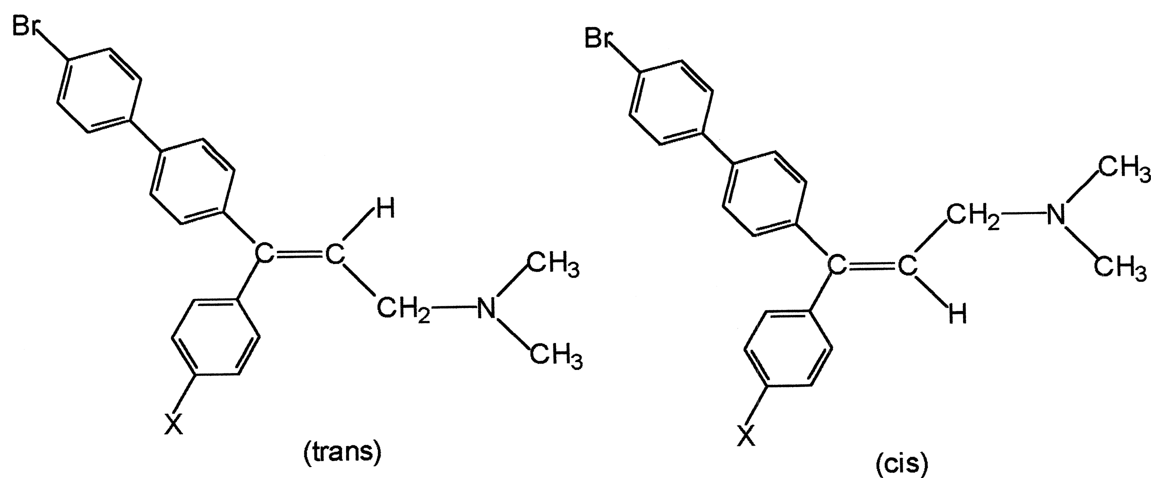

Fig. 1. Chemical structures of I (where X=H) and internal standard II (where X=NO_2).

purposes, despite of the *Z/E* designations of Cahn–Ingold–Prelog, when the larger groups are on the same side of the double bond plane, it will be referred to as *cis*-isomers, and when those groups are on opposite sides of the double bond plane, it will be referred to as *trans*-isomers.

Differences in the biological properties of the individual stereoisomers have indicated that the *cis*-isomer is consistently more active against the parasite in vitro [11,12], but more toxic to mammalian and bacterial cells [9,10,12,13], when compared to the *trans* ones.

The unsubstituted (where X=H, compound I) derivative, which exhibited high chemotherapeutic activity against *T. cruzi* in vitro, is considered the prototype compound of this series, and for this reason was chosen for metabolism studies. In this report, an efficient and reproducible HPLC method for determination of *cis*- and *trans*-isomers of I in rat feces is described for the first time. Also, this method was applied to pilot experiments in rats, revealing that considerable amounts of I, in the unchanged form, are excreted in the feces from dosed rats.

2. Experimental

2.1. Reagents and chemicals

All solvents used in the HPLC system were of analytical grade and those used in synthesis and purification were of reagent grade. Unsubstituted (I) and nitro (II) diarylpropenamine derivatives, 3-(4′-bromo-[1,1′-biphenyl]-4-yl)-3-(4-X-phenyl)-N,N-dimethyl-2-propen-1-amine, (where X=H or NO_2, respectively) were synthesized in our laboratory and purified in order to achieve high purity [9]. The isomeric proportion in the synthetic product of I is 50% for *cis*-isomer and 50% for *trans* one (assessed by ^{1}H NMR, 300 MHz). Compound II, used as internal standard, was kindly supplied by Dr. Roseli De Conti. Both compounds were fully characterized by infrared, ^{1}H NMR and mass spectrometry. Bond Elut Jr (500 mg) cartridges were purchased from Varian (Harbor City, CA, USA).

2.2. Apparatus and chromatographic conditions

The HPLC apparatus consisted of a Shimadzu series 10A (Kyoto, Japan) solvent delivery pump and an UV spectrophotometric detector Model SPD-10A which was set at 272 nm. Separations of the geometrical isomers of I were achieved on a normal-phase Nova-Pak silica column (150×4.6 mm I.D.; 5 μm particle size; Kyoto, Japan).

The mobile phase consisted of ethyl acetate–*n*-hexane–triethylamine (59:40:1, v/v/v) at a flow-rate of 1 ml/min. The mixture was filtered through a 0.22 μm Nylon 66 membrane and degassed before use. Sample preparation and analysis were performed at ambient temperature.

2.3. Preparation of calibration standards and quality control samples

Stock solutions were prepared by accurately weighing the appropriate amounts of I and internal standard and dissolving each separately in ethyl acetate in 5 ml volumetric flask to prepare 10 mg/ml stock standard solutions. Standard solutions of I were prepared from the stock solution by sequential dilutions with ethyl acetate to give five concentrations of 100, 200, 500, 1000 and 1500 μg/ml (each isomer corresponding to half of these concentrations). Working internal standard solution was prepared by diluting II with ethyl acetate to give concentration of 500 μg/ml.

Quality control feces samples were prepared at three concentrations (10, 50 and 150 μg/g feces) by adding 0.1 ml of working solutions to approximately 0.5 g of rat feces. Quality control feces samples were previously homogenized by suspending drug-free rat feces (approximately 15 g) in 50 ml of distilled water and submitting it to natural evaporation.

2.4. Feces samples preparation

Calibration standards, quality control and unknown feces samples were processed by a solid-phase extraction procedure on Bond Elut Jr silica cartridges. The cartridges were first conditioned with 3.0 ml of ethyl acetate followed by 5.0 ml of hexane under atmospheric pressure.

Samples of spiked feces (approximately 0.5 g), standard solution (0.1 ml), internal standard solution (0.1 ml) and 2 ml of 1 M carbonate buffer (pH 9.4) were combined in a glass tube and vortex mixed for 1 min. After addition of 3 ml of ethyl acetate, the mixture was vortex mixed for 30 s at high speed and centrifuged at 1430 *g* for 10 min. The supernatant was transferred into a glass tube and loaded onto a pre-activated cartridge. After the cartridge was washed with 5.0 ml of hexane, the analytes were eluted with 5.0 ml of ethyl acetate–methanol (1:1, v/v) into a glass tube. The eluent was evaporated to dryness under vacuum at 40°C and then reconstituted in 200 μl of the mobile phase. An aliquot of 50 μl of the reconstituted extract was injected into the HPLC system.

For determination of the drug concentration in samples from dosed rats, 0.1 ml of internal standard solution and 2 ml of 1 M carbonate buffer (pH 9.4) were combined in a glass tube and submitted to the same analytical procedure describe above.

2.5. Validation procedures

Drug-free feces, spiked with known amounts of I, were analyzed according to the respective analytical method described above. Peak-area ratios of *cis*- and *trans*-I to II were plotted against the respective concentrations to give the calibration graphs.

Intra-day precision of the calibration standard was determined from the coefficient of variation of back-calculated at low, medium and high concentration level. Five sets of each concentration (10, 50 and 150 μg/g) of *cis*- and *trans*-I were analyzed on the same day. Accuracy was tested simultaneously with precision and was expressed as the percentage deviation of the mean concentration found from the nominal one. Inter-day accuracy and precision of the method were also determined simultaneously by replicate measurements ($n=3$) of quality control samples on three days.

The percentage recoveries of extraction for each compound from feces were calculated from the peak-area ratios of spiked samples relative to directly injected standard solutions.

2.6. Animals

For metabolism studies, fifteen adult male Wistar rats weighing 230–250 g (Central Bioterium, UNICAMP, Brazil) were randomly placed in individual metabolic cages with water and food ad libitum. They were dosed either orally by gavage ($n=5$) or intraperitoneally ($n=5$) with 50 mg/kg body weight of I (as free base) suspended in 12% (w/v) Tween 80 aqueous solution (treated group). The control group ($n=5$) received vehicle only. After administration, food was withheld for 48 h and then supplied from 48–96 h. At all times the rats were allowed access to water. Feces were collected for 96 h in two pools (0–48 h and 48–96 h) and stored at −18°C until worked up.

3. Results and discussion

3.1. Chromatography

Fast and reproducible separation of the analytes was achieved using a silica normal-phase column, eluted isocratically with ethyl acetate–*n*-hexane–triethylamine (59:40:1). The use of solid-phase extraction procedure with silica cartridges led to chromatograms relatively free of endogenous interferences from the biological matrix, compared with liquid–liquid procedures employed previously. The combination of both extraction procedures was required to eliminate the interferences of the retention time of each compound on the chromatogram. Fig. 2 shows representative chromatograms obtained from analysis of blank rat feces sample and feces sample from rats treated either orally or intraperioneally with I, containing compound II at microgram levels used as an internal standard. The peaks of both *cis*- and *trans*-isomers were well resolved and their symmetry was good. The retention times were 7.2 min for *cis*-isomer and 8.1 min for *trans* one, and their resolution factor was 1.34. The identity of both isomers was unambiguously assessed by comparing their retention times with those of pure and well-characterized (by ^{1}H NMR) individual isomers. In addition, each peak was collected and their UV-spectra determined. The shorter wavelength, 264 nm, was attributed to *cis*-isomer and the longer wavelength, 286 nm, to the *trans* one. These attributions were based on the conformational effects on electronic spectra of the conjugated diarylpropenamine system. In the *cis*-conformation, bulky groups interaction results in distortion of the coplanarity of the π-system and in turn in higher energy required to excite ground-state electrons. In the *trans*-conformation, however, this effect is not pronounced, and a lower electronic energy is observed.

3.2. Assay linearity, accuracy, precision and sensitivity

The calibration graphs were linear over the concentration range 10–150 μg/g, which provided typical linear regressions of $y=0.00532x+0.0101$ ($R=0.9992$) for *cis*-I and $y=0.00469x+0.0138$ (R=0.9994) for *trans*-I.

The recoveries of I and II from spiked feces samples were calculated by comparing the peak area of extracted samples at low, medium and high concentration levels, with those obtained from the analysis of corresponding directly injected standards ($n=3$). The average recoveries of *cis*-I, *trans*-I and II were 55.3±6.2, 51.7±4.9 and 51.0±15.2%, respectively. These losses in recovery are probably consequence of the two-steps clean-up procedure.

The reproducibility of the method was evaluated by analyzing replicates of feces quality control samples containing low, medium and high concentrations levels of I. Table 1 shows the precision and accuracy based on back-calculated values of quality control samples measured on three days, with five or three samples at each concentration. The precision (coefficient of variation) of the method in rat feces was within acceptable limits (less than 15%) both for intra- and inter-days. The percent accuracy was also within ±15% deviation of the nominal values.

The detection limit of the assay, defined as the smallest concentration that can be distinguished from the noise level, was found to be ca. 2 μg/g for compound I in feces samples.

3.3. Stability of compounds I and II

Standard solutions and spiked samples frozen in aliquots were stable for at least 6 months, since no changes in I or II concentrations were detected during this period. No degradation product was also detected in reconstituted extracts for at least 24 h after analysis.

3.4. Application of the method to feces samples

The main routes of drug excretion in quantitative terms are the renal and the biliar (hepatic) vias. The renal excretion is particularly important in the elimination of more polar compounds and drug metabolites. Substances excreted in the feces are mainly unabsorbed orally ingested drugs or those which are excreted in the bile and are not reabsorbed by the gut [14].

The presence of I in both biological materials was investigated. In urine samples, neither the parent compound nor its possible metabolites or conjugated forms have been detected yet, despite performing

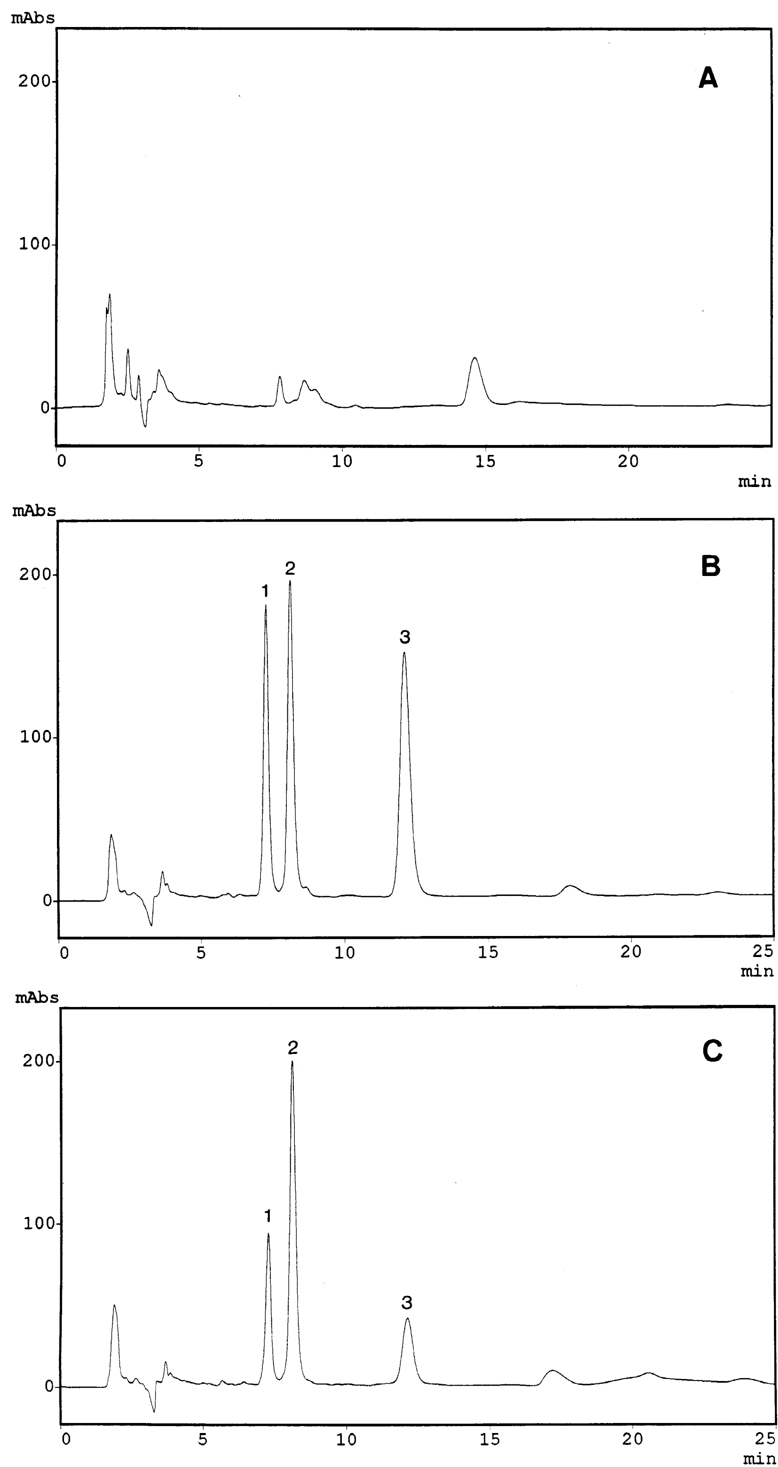

Fig. 2. Representative HPLC chromatograms of fecal extracts: (A) extracts of samples from control (drug-free) rats; (B) sample obtained from rats after a single oral dose of 50 mg/kg of I; (C) extracts of sample from rats receiving a single intraperitoneal dose of 50 mg/kg of I. Peak 1=*cis*-I; Peak 2=*trans*-I; Peak 3=internal standard II.

Table 1
Intra- and inter-day precision and accuracy for *cis*- and *trans*-I in feces

Concentration (μg/g)		Coefficient of variation (%)	Deviation[a] (%)
Added	Found (Mean±SD)		
Intra-day reproducibility ($n=5$)			
cis-I			
10	11.11±1.25	11.25	11.10
50	46.49±4.23	9.10	−7.02
150	151.79±11.30	7.44	1.19
trans-I			
10	10.57±0.87	8.23	5.70
50	49.49±6.28	12.69	−1.02
150	155.57±10.96	7.04	3.71
Inter-day reproducibility ($n=3$)			
cis-I			
10	11.26±0.84	7.46	12.60
50	47.06±4.97	10.56	−5.88
150	155.86±11.01	7.06	3.91
trans-I			
10	10.41±1.25	12.01	4.10
50	49.87±6.40	12.83	−0.26
150	157.89±15.15	9.59	5.26

[a] Deviation=[(found concentration−added concentration)/added concentration]×100.

intensive investigations, including enzymatic (using β-glucuronidase) and acid hydrolyses of the samples (data not shown). The high hydrophobicity of I combined with its possible slow metabolism rate may explain this finding. On the other hand, this compound was detected in considerable amounts, only in the unchanged form, in feces samples.

In order to determine *cis*- and *trans*-isomers of I in feces samples, the present HPLC method was developed and applied to pilot experiments in rats. Table 2 shows concentrations of I determined from two pools (0–48 h and 48–96 h) of samples feces from rats treated either orally or intraperitoneally with 50 mg/kg of I.

The presence of the stereoisomers of I was also confirmed in thin-layer chromatography. When analyzed in silica gel analytical plates (5×20 cm laboratory-prepared; 0.25 mm thickness; mobile phase: ethyl acetate–methanol, 92:8, v/v), both *cis*- and *trans*-isomers were visualized under the UV light (wavelength 254 nm) in the treated group (R_F 0.11 and 0.14, respectively).

The presence of I in sample feces after intraperitoneal administration indicates that this compound is excreted in the bile by the liver and also excludes the hypothesis that I is orally unabsorbed. Giving its lipid-soluble character, we presume this compound would be reabsorbed from the intestinal tract, and then be particularly subjected to the enterohepatic cycle. The effect of this cycle is to

Table 2
Concentrations of *cis*- and *trans*-I found in feces from treated rats ($n=2$)

Route of administration	Concentration found (μg/g)	
	0–48 h (pool 1)	48–96 h (pool 2)
Oral		
cis-I	162±8	ND[a]
trans-I	238±17	ND
Intraperitoneal		
cis-I	57±9	285±16
trans-I	132±20	771±70

[a] ND=not done.

increase the persistence of the drug in the body. Previous work supports this assumption. The *para*-chloro (where *X*=Cl) diarylpropenamine analogue, which has log *P* comparable to that of I (6.25 and 6.96, respectively), revealed to be well orally absorbed and to have a relatively long half-life and significant accumulation in tissues [15].

It is interesting to note that *trans*-I isomer is excreted in around 1.5–2.5 fold higher amounts in feces samples than its *cis* counterpart, for oral and i.p. routes, respectively (see Table 2), meaning that a difference in the pharmacokinetic profiles exists between these two isomeric forms. The reason for this difference, however, is not yet known, since insufficient information about their absorption, metabolism and distribution is available.

4. Conclusion

The chromatographic system described here has been shown to be applicable to the determination of the *cis*- and *trans*-isomers of I at microgram levels in feces, using a solid-phase extraction procedure. The method is specific and reproducible and could be useful in further investigations on the pharmacokinetics of its geometrical isomers in preclinical studies. Based on our results, it could be anticipated that fecal excretion is quantitatively the major route for I elimination.

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