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Short communication

The identification of (−)-*trans*-tadalafil, tadalafil, and sildenafil in counterfeit Cialis® and the optical purity of tadalafil stereoisomers

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ABSTRACT

Four blisters with suspect Cialis® (tadalafil) 20 mg tablets were screened for authenticity using near infrared spectroscopy (NIRS) and for the presence of phosphodiesterase 5 (PDE-5) inhibitors using LC-DAD–MS. All samples were identified as counterfeit Cialis® and contained sildenafil or a combination of tadalafil and sildenafil. Although the tablets contained efficacious amounts of PDE-5 inhibitors, neither the active ingredient nor the dosage corresponded to the description on the blister. This is the first reported case of a diastereomeric mixture of tadalafil and *trans*-tadalafil (3:1) being identified in a counterfeit medicine. The LC-DAD-CD revealed that both diastereomers had a high optical purity. The optical rotation of the diastereomeric mixture was measured indicating the presence of (−)-*trans*-tadalafil, which is the only other stereoisomer with some PDE-5 inhibitory activity.

As no safety profiles are known for the stereoisomers of tadalafil, there is a potential health risk. In addition, the optical purity of tadalafil needs to be taken into account when calculating the dosage in illegal medicines.

1. Introduction

The introduction of Viagra® (sildenafil citrate) as a treatment for erectile dysfunction (ED) quickly resulted in its misuse as a supposed aphrodisiac [1]. Since then, sildenafil and many structural analogues described in the Pfizer patents have been detected in counterfeit Viagra® and in adulterated dietary supplements [2–4]. The same fate has struck the other two FDA and EMEA approved ED drugs: Cialis® (tadalafil) and Levitra® (vardenafil HCl $3H_2O$) [5].

The visual similarity of the counterfeit ED drugs (tablets, blisters, boxes) has improved over the last decade [6]. Advanced techniques, such as near infrared spectroscopy (NIRS), have proved useful [7,8] in rapidly distinguishing between counterfeit and authentic tablets. However, investigators still rely on identification and quantification of the active ingredients when assessing acute health risks.

Of the three phosphodiesterase 5 (PDE-5) inhibitors mentioned, tadalafil is the only optically active compound. Tadalafil contains two chiral centres in the 6R, 12aR configuration, which is the (+)-*cis* stereoisomer (Fig. 1) [9]. Of the three other stereoisomers, only (−)-*trans*-tadalafil shows some PDE-5 inhibitory activity, whereas none of the stereoisomers should be present in Cialis®. Although tadalafil is regularly identified in counterfeit medicines, no investigation into the presence of stereoisomers has to our knowledge as yet been reported. As none of the stereoisomers have been clinically evaluated for safety, there is an inherent health risk.

The present study describes four suspect blisters of Cialis® tablets that were brought in for analysis by the Netherlands Health Care Inspectorate. Our laboratory was asked to verify the authenticity of the tablets and to identify and quantify any active pharmaceutical ingredients (APIs) used. After applying our standard analysis methods, the presence of a tadalafil diastereomer was suspected in one sample. Consequently, the structure of this component was elucidated and the optical purity of the samples with tadalafil was assessed.

2. Materials and methods

The four Cialis® blister packs brought in for analysis each contained two characteristically pear-shaped yellow tablets with "C20" embossed on either side. The expiration date and lot numbers declared on the blister packs were recorded. The samples were then assigned the letters A to D.

2.1. NIRS

The authenticity of Cialis® tablets was verified in our laboratory by using near infrared spectroscopy (NIRS). The methods used for the authentication were identical to those described earlier for

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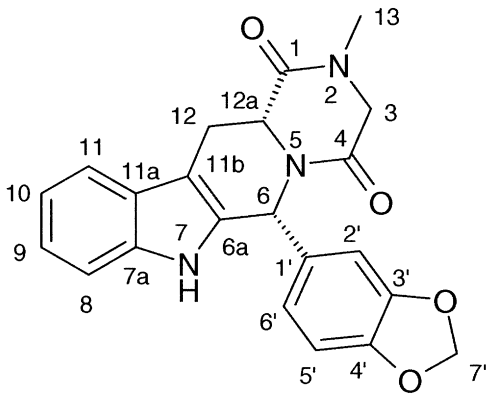

Fig. 1. The numbering of tadalafil atoms.

the authentication of Viagra® tablets [7]. However, in this case the screening method was used to detect counterfeit Cialis® tablets using a reference library of authentic Cialis® [8]. This reference library was constructed by recording the spectra of both sides of five tablets of five different batches of authentic 20 mg Cialis® tablets. After validating the library, a threshold of 0.998 was defined for wavelength correlation (WC). Higher values indicated that a tablet could not be distinguished from authentic Cialis®. This criterion was set for both a single tablet and for an entire sample. All suspect tablets were measured on both sides and all spectra were compared to the library of authentic tablets.

2.2. LC-DAD–MS^n

For the identification and quantification of ED drugs the samples were analyzed by LC-DAD–MS^n using our standard method described earlier [2]. One tablet per blister was powdered and about one dosage unit was sonicated in 100 mL of MeOH for 20 min, followed by centrifugation at 3200 rpm for 5 min. For screening, an aliquot (5.0 mL) of the supernatant liquid was diluted 20× using eluent. The stock solution was used for the quantification of low dosages. All samples and solutions were filtered before use over 0.45 μm Spartan 30 filter (Whatman GmbH, Dassel, Germany). For the separation of tadalafil and the unknown component, the same method was used but with a symmetry C_{18} 2.1 mm × 100 mm column with a 3.5-μm particle size (Waters Corp., Milford, MA, USA) and 20 min elution time.

2.3. LC–UV–CD

An LC–UV system that could be coupled to a circular dichroism (CD) detector was used to investigate the optical rotation of tadalafil and the suspected tadalafil diastereomer. We used an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a binary pump, in-line degasser, thermostated autosampler, and a UV detector. The HPLC was coupled to a Jasco CD-1595 detector. UV- and CD-detection was performed at 284 nm (UV_{max} for tadalafil). Chromatography was performed on a symmetry C_{18} 3.9 mm × 150 mm column with a 5-μm particle size (Waters Corp., Milford, MA, USA). The mobile phase used was a 1:1 mixture of methanol (B) and water with 0.1% formic acid (v/v) buffered at pH 4 using conc. ammonia (A). The flow rate was set at 1 mL/min and isocratic elution was employed for 15 min. The injection volume was 20 μl using the samples and calibration curve prepared above. The optical purity was assessed by comparing the dosages calculated for the UV and CD signal using the tadalafil calibration curve. The optical purity of reference tadalafil was >99.9% (Lilly ICOS LLC, Indianapolis, IN, USA).

2.4. ^1H and ^{13}C NMR

In order to confirm the structure of the suspected tadalafil diastereomer, the diastereomer was isolated as a mixture together with tadalafil. The ^1H NMR and ^{13}C NMR spectra of the mixture were recorded as described earlier [10]. The stock solution of sample A in MeOH was filtered and 50 mL of the filtrate was evaporated under vacuum. The resulting solid was washed with $CHCl_3$ (3× 20 mL). The combined washings were filtered and evaporated yielding 10 mg of an off-white solid. For ^1H NMR and ^{13}C NMR analysis, 2.5 mg of the solid was dissolved in $CDCl_3$.

2.5. Polarimeter

To determine the presence of (+)- or (−)-*trans*-tadalafil in sample A, the optical rotation of the diastereomeric mixture isolated for NMR was measured using a PerkinElmer 241b polarimeter at 20 °C with a 100 mm × 3 mm cell (1 mL). Literature states that the specific rotation is +71° (c 1.13, $CHCl_3$) for tadalafil, −71° (c 1.02, $CHCl_3$) for (−)-tadalafil, +297° (c 1.21, $CHCl_3$) for (+)-*trans*-tadalafil and −293° (c 1.28, $CHCl_3$) for (−)-*trans*-tadalafil [9]. The 4° discrepancy observed for the *trans*-enantiomers was not explained.

Because part of the isolated mixture had to be retained as evidence in a court case, it was considered not feasible to isolate *trans*-tadalafil and measure the optical rotation. Therefore, the optical rotation of sample A and the reference tadalafil were measured at a concentration lower than reported in literature (c 0.1, $CHCl_3$). Excipient effects needed to be accounted for as optically active excipients, such as lactose and cellulose (e.g. present in authentic Cialis® tablets) [11], may have been isolated. For this, the ^1H NMR of sample A was checked for impurities. In addition, samples C, D and an authentic Cialis® 20 mg tablet (=AT) were also prepared for measurement. An AT stock solution in MeOH was prepared as described above. Subsequently, 50 mL of the filtered stock solution of samples C, D and AT in MeOH was evaporated and the residue of each sample was dissolved in sufficient $CHCl_3$ to reach (c 0.1). The average of the triplicate measurements was reported.

3. Results and discussion

3.1. NIR spectroscopy

None of the spectra of suspect tablets exceeded the threshold for WC using our library of authentic Cialis® 20 mg tablets. Correlation factors are listed in Table 1. As all the samples differed significantly from the authentic tablets, they were considered to be counterfeit Cialis® 20 mg tablets. The spectra of tablets in each blister pack could not be distinguished from each other.

3.2. LC-DAD–MS^n

Fig. 2 shows the DAD-trace (250–450 nm) of the chromatograms for both the standard mixture and the four suspect samples. The identity of tadalafil and sildenafil was confirmed by retention time, UV-spectra, MS^1 and ion ratios in MS^2. A five-point calibration curve was used for quantification of the DAD-signal at the spectrum maximum: sildenafil (λ_{max} = 291 nm); range: 1.05–10.1 μg/mL, precision $RSD_{sildenafil}$ = 0.8%, linearity R^2 = 0.9999, $y = 661595x - 31.43$: tadalafil (λ_{max} = 284 nm); range: 2.44–20.7 μg/mL, precision $RSD_{tadalafil}$ = 0.8%, linearity R^2 = 0.9996, $y = 726875x - 181830$.

Sildenafil was identified in every sample, even though it should not be present in Cialis® (Table 1). Pharmacologically relevant quantities were only found in sample B. The sub-therapeutic quantities of sildenafil found in samples A, C, and D suggest poor production hygiene.

Table 1
Analysis results of the four suspect Cialis® 20 mg samples.

Sample	Lot no./exp.	NIR corr.[a]	API identified	Dose[b]		
				LC-DAD–MS	LC–UV–CD	LC–UV–CD
A	Lot 05668 Exp: 01, 2009	0.8979	Tadalafil (−)-*trans*-Tadalafil Sildenafil	18.0 mg[c] Traces	14.6 mg 3.6 mg[d] nd	14.5 mg 3.5 mg[d] [4.8 mg[e]] nd
B	A 47485 03.08.2012	0.9003	Sildenafil	58.1 mg	nd	nd
C	Lot 05668 Exp: 04, 2011	0.7983	Tadalafil Sildenafil	11.1 mg 1.0 mg	11.2 mg nd	11.1 mg nd
D	Lot 05668 Exp: 04, 2011	0.8118	Tadalafil Sildenafil	12.0 mg 1.1 mg	12.1 mg nd	12.0 mg nd

nd: not done.
[a] A correlation <0.998 is considered significantly different from authentic.
[b] The signal used for quantification per method is underscored.
[c] Peaks not separated; dose calculated for the total of tadalafil stereoisomers using the response factor for tadalafil.
[d] Calculated using response factor for tadalafil.
[e] Adjusted dose using the *cis*/*trans* ratio found by ^{1}H NMR.

Tadalafil was identified in samples A, C and D in pharmacologically relevant quantities. Although sharp peaks were observed for tadalafil in the standard mixture and in samples C and D, a shoulder was observed for the tadalafil signal sample A (Fig. 2). No significant differences from standard tadalafil were observed in UV_{max} (<2 nm), MS^1 (full scan) or MS^2 at any point in the peak.

Using the same method, but with the symmetry column and 20 min elution, resulted in the separation of the peaks, the largest peak representing tadalafil (Fig. 3). The UV_{max} of the two separate peaks differed by 2 nm (Table 2). A full scan MS^1 showed a difference in ionisation between the peaks. Contrary to tadalafil the unknown component showed a significant $2M+H^+$ signal implying

Table 2
DAD and MS results for tadalafil and the unknown in sample A.

Component	UV_{max}	MS^1 (*m*/*z*)	MS^2 (ion ratio)	
Tadalafil	291	390 ($M+H^+$)	302 (23%)	262 (100%)
	284		273 (19%)	250 (47%)
	231		268 (56%)	135 (51%)
trans-Tadalafil (the unknown)	289	390 ($M+H^+$)	302 (23%)	262 (100%)
	282	779 ($2M+H^+$)	273 (19%)	250 (46%)
	231		268 (53%)	135 (51%)

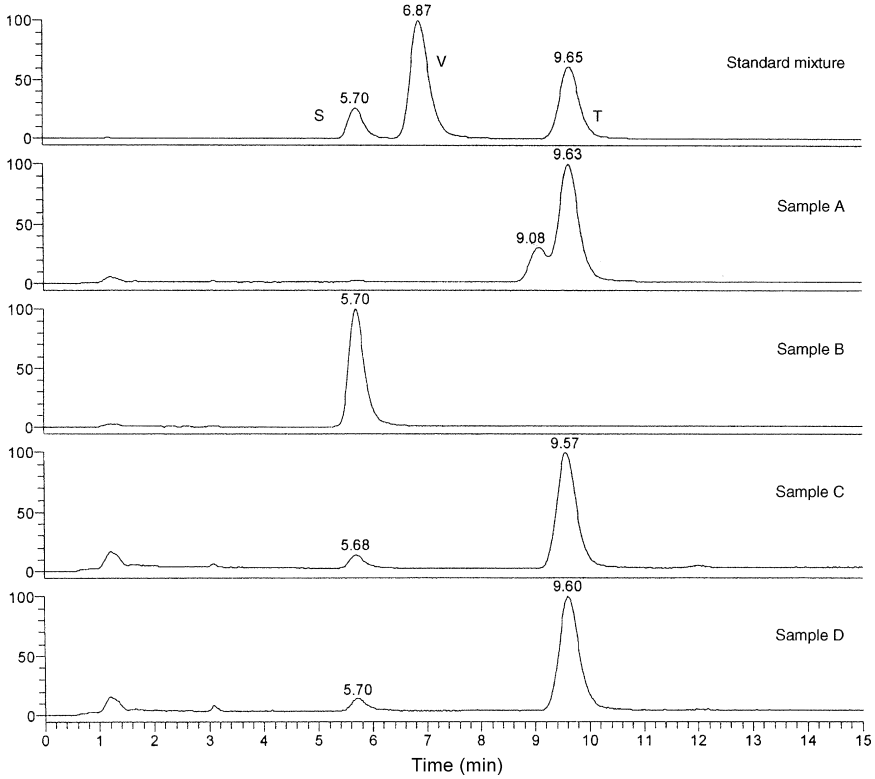

Fig. 2. PDA traces of the standard mixture of sildenafil (S), vardenafil (V), tadalafil (T) and the suspect Cialis® 20 mg samples using an XTerra MS C_{18} column (100 mm × 2.1 mm, 3.5 μm).

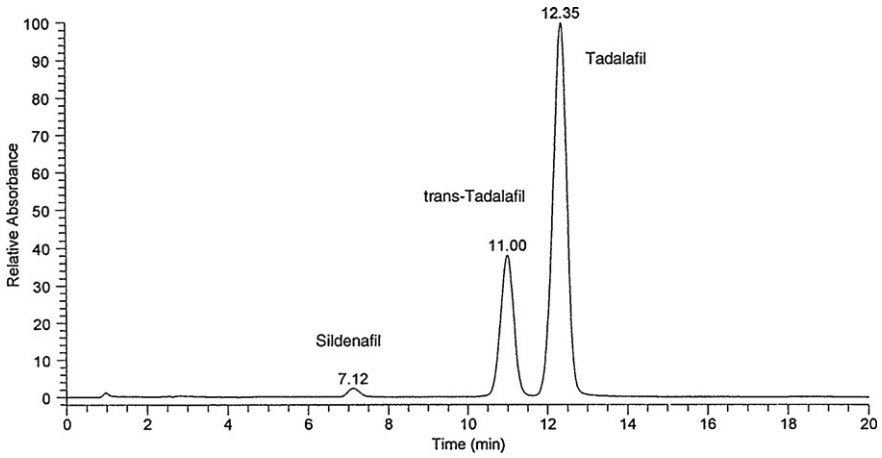

Fig. 3. PDA trace for sample A using the LC-DAD–MS method using a symmetry C_{18} column (100 mm × 2.1 mm, 3.5 μm).

a structural difference. However, the two components could not be distinguished from the MS^2 spectra.

Taking the two chiral centres present in tadalafil into consideration, the possible presence of diastereomers and enantiomers was investigated on a LC–UV system connected to a circular dichroism (CD) detector. None of the stereoisomers were available as a reference standard.

3.3. LC–UV–CD

The separation method was transferred to a LC–UV system that could be coupled with a CD-detector. Tadalafil and the unknown component in sample A produced a CD signal oriented in the same direction as the reference tadalafil (Fig. 4). The same direction of rotation was observed for tadalafil in samples C and D. The tadalafil calibration solutions prepared above were used in this method: precision RSD_{UV} = 2.5%, linearity R^2 = 0.9994, $y = 41.752x - 36.405$; precision RSD_{CD} = 3.7%, linearity R^2 = 0.9989, $y = 1586x - 1.820$. Dosages calculated by either the UV- or the CD signal were in agreement, implying high optical purity (Table 1). Considering the precision of the CD signal, the optical purity for tadalafil in samples A, C and D and for the unknown component were estimated to be >96%. The dosage of the unknown component was estimated using the UV- and CD-response of tadalafil at 284 nm.

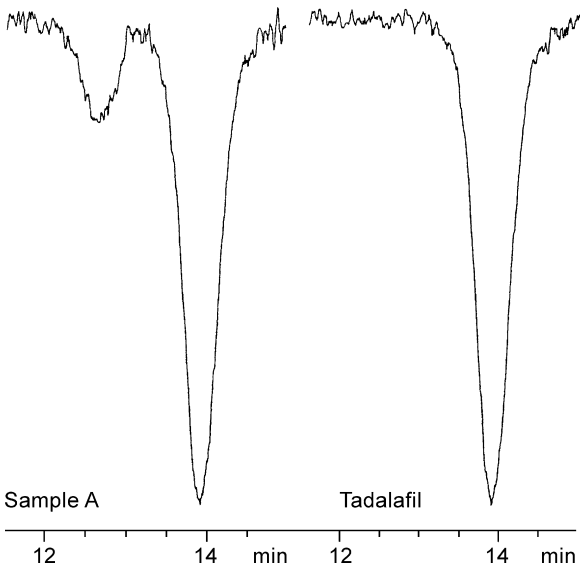

Fig. 4. CD signals for sample A and reference tadalafil (284 nm).

The observed direction of rotation for the unknown component could not be linked to either a (+)- or (−)-*trans*-tadalafil, because its optical rotation at 284 nm has not been described in literature.

3.4. ^1H and ^{13}C NMR

The ^1H and ^{13}C NMR data on sample A confirm the presence of a mixture of tadalafil and *trans*-tadalafil (Table 3) [9]. The *trans*-C_6H could not be assigned due to overlapping signals at the expected chemical shift (≈6.8 ppm). Using the free lying $C_{7'}H$ signals, the actual tadalafil: *trans*-tadalafil ratio was calculated to be 3:1 (instead of the 4:1 ratio by UV). The dosage estimated using the UV response of tadalafil was therefore recalculated at 4.8 mg (Table 1).

The synthesis of tadalafil reported in literature describes (−)-tryptophan methyl ester being converted to a 1:1 mixture of a *cis*- and *trans*-intermediate [9,12]. The (+)-*trans*-intermediate is a side-product, which is either separated or can be isomerised *in situ* to specifically yield the (+)-*cis*-intermediate [13]. The (+)-*cis*-intermediate is required for the synthesis of tadalafil. Continuing synthesis with the mixture of intermediates eventually results in the co-formation of (+)-*trans*-tadalafil, which is the 6S, 12aR configuration. Starting from (+)- or (±)-tryptophan methyl ester eventually results in the formation of the corresponding (−)-enantiomers or the racemate. According to literature, (−)-*trans*-tadalafil is the only other tadalafil stereoisomer with some PDE-5 inhibitory activity [9].

3.5. Polarimeter

Because the diastereomers in sample A were found to be enantiomerically pure by LC–UV–CD, the observed optical rotation is the net result of the contribution of both diastereomers. Ideally, assuming proportional contributions, the optical rotation for a 3:1 diastereomeric mixture would show α_D = +128° for tadalafil and (+)-*trans*-tadalafil and α_D = −20° for tadalafil and (−)-*trans*-tadalafil. Reference tadalafil showed an optical rotation of α_D = +71° (c 1.00, $CHCl_3$), which was in agreement with the literature [9]. No significant concentration dependency was observed for reference tadalafil (α_D = +72°, c 0.10, $CHCl_3$) and no significant excipient effects were observed for samples C (α_D = +71°), D (α_D = +71°) and AT (α_D = +73°) (all: c 0.10, $CHCl_3$). From the ^1H NMR there were no

Table 3
^{1}H and ^{13}C NMR data of the mixture of tadalafil and *trans*-tadalafil in sample A.

Atom no.	nH	Tadalafil		*trans*-Tadalafil	
		^{1}H(δ)	^{13}C(δ)	^{1}H(δ)	^{13}C(δ)
1			166.8		165.5
3	2	3a: 3.94 (d 17.6 Hz, 1H) 3b: 4.10 (dd 17.4 Hz, 1.5 Hz, 1H)	52.1	3a: 3.99 (d 17.6 Hz, 1H) 3b: 4.14 (dd 17.9 Hz, 1.5 Hz, 1H)	51.5
4			166.4		161.5
6	1	6.14 (s, 1H)	56.8	≈6.8, not seen due to overlapping signals	52.4
6a			132.7		129.7
7	1	7.77 (s, 1H)		7.83 (s,1H)	
7a			136.5		136.3
8	1	8–10: 7.32–7.12 (m, 3H)	111.2	8–10: 7.33–7.23 (m, 3H)	111.1
9	1		122.6		122.8
10	1		120.2		120.1
11	1	7.60 (d 6.6 Hz, 1H)	118.7	7.53 (d 7.7 Hz, 1H)	118.5
11a			126.2		126.3
11b			106.7		108.3
12	2	3.21 (ddd 16.1 Hz, 11.7 Hz, 1.5 Hz, 1H) 3.78 (dd 16.1 Hz, 4.4 Hz, 1H)	23.9	2.95 (dd 15.4 Hz, 1.5 Hz, 1H) 3.55 (dd 15.4 Hz, 4 Hz, 1H)	22.7
12a	1	4.30 (m, 1H)	56.2	4.36 (m, 1H)	51.8
13	3	3.04 (s, 3H)	33.6	2.99 (s, 3H)	33.4
1′			135.3		132.0
2′	1	6.73 (d 1.8 Hz, 1H)	107.4	6.72 (m, 1H)	109.2
3′			147.9		148.2
4′			147.1		148.0
5′	1	6.69 (d 8.1 Hz, 1H)	108.2	6.81 (s, 1H)	109.2
6′	1	6.86 (dd 8.1 Hz, 1.8 Hz, 1H)	120.8	6.98 (s, 1H)	122.5
7′	2	5.87 (AB, 9.4 Hz, 2H)	101.1	5.93 (s, 2H)	101.4

indications of the presence of impurities in the isolated diastereomeric mixture.

The optical rotation of sample A was $\alpha_D = -25^\circ$, which is close to the ideal value for a 3:1 mixture of tadalafil and (−)-*trans*-tadalafil. Deviations from the theoretical value may be caused by a non-proportionality, concentration effects, the 4° discrepancy described earlier, and excipient effects. Based on the ^{1}H NMR results and the results for samples C, D and AT, significant excipient effects are unlikely but cannot be ruled out entirely.

4. Conclusion

NIRS and LC-DAD–MS analysis have shown the four suspect Cialis® samples investigated in this study to be counterfeit and substandard medicines. All counterfeits and contained sildenafil or a combination of tadalafil and sildenafil. Although the tablets contained efficacious amounts of PDE-5 inhibitors, neither the active ingredient nor the dosage corresponded to the description on the blister. The low level presence of sildenafil in three of the samples is evidence of poor production hygiene.

This is the first time that a diastereomer of tadalafil has been identified in counterfeit medicine. In addition, the optical purity of tadalafil in counterfeit medicine was investigated. Tadalafil was shown to be optically pure in samples A, C and D, as well as the *trans*-tadalafil in sample A. The optical rotation of the diastereomeric mixture indicates the presence of (−)-*trans*-tadalafil, which is about 20 times less potent than tadalafil. As (−)-*trans*-tadalafil is not a side-product in the synthesis of tadalafil, it would have been added deliberately.

The content of the counterfeit Cialis® tablets investigated in this study has proved to be unreliable. The presence of low levels of sildenafil suggests that these are impurities resulting from poor manufacturing practice. The presence of *trans*-tadalafil is worrying because safety and toxicity profiles are lacking. Therefore, in the future, the presence of stereoisomers needs to be investigated in order to be able to accurately report dose and assess health risks.

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