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Liquid chromatographic method for the simultaneous determination of achiral and chiral impurities of dapoxetine in approved and counterfeit products

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ABSTRACT

A reversed-phase high performance liquid chromatographic method was developed and validated for the simultaneous determination of the related substances of *S*-dapoxetine, including *R*-dapoxetine, (3*S*)-3-(dimethylamino-3-phenyl-1-propanol), *S*-3-amino-3-phenyl-1-propanol, 1-naphtol, 4-phenyl-2H,3H,4H-naphtho[1,2-*b*]pyran and 1-(2*E*)-Cinnamyloxynaphthalene. During the screening experiments seven different polysaccharide-type chiral stationary phases (amylose-based Lux-Amylose-1, Lux-i-Amylose-1 and Lux-Amylose-2, as well as cellulose-based Lux-Cellulose-1, Lux-Cellulose-2, Lux-Cellulose-3 and Lux-Cellulose-4) were tested in polar organic mode using a mobile phase consisting of 0.1% diethylamine in methanol, ethanol, 2-propanol and acetonitrile with 0.5 mL min^{-1} flow rate at 20 °C. Best results were obtained on Lux Cellulose-3 column with the ethanol-based mobile phase. To increase the retention factor of two, early-eluting impurities, water was added to the mobile phase. In order to counterbalance the increased total analysis time, higher column temperature (40 °C) and gradient elution, combined with flow-programming' was applied. Using the optimized conditions baseline separations were achieved for all compounds within 30 min. The method was validated according to the International Council on Harmonization guideline Q2(R1) and applied to the analysis of an approved, tablet formulation and dapoxetine-containing products sold on the internet. As expected, in the case of the pharmacy-acquired product, all of the monitored impurities were below 0.1%. However, interesting results were obtained when internet-acquired samples were analyzed. These tablets contained racemic dapoxetine and/or high concentration of *R*-dapoxetine impurity. Based on this work polysaccharide-based chiral stationary phases can be successfully applied for the simultaneous determination of achiral and chiral impurities in reversed-phase mode applying gradient elution and flow-rate programs. The study further underlines the importance of not only achiral, but also enantiomeric quality control, whenever counterfeiting of a single enantiomeric agent is suspected.

1. Introduction

Premature ejaculation (PE) is one of the most common sexual disorders among men. It is characterized by short ejaculatory latency (≤ 1 min), a lack of control over ejaculation and associated negative personal consequences [1]. The condition affects

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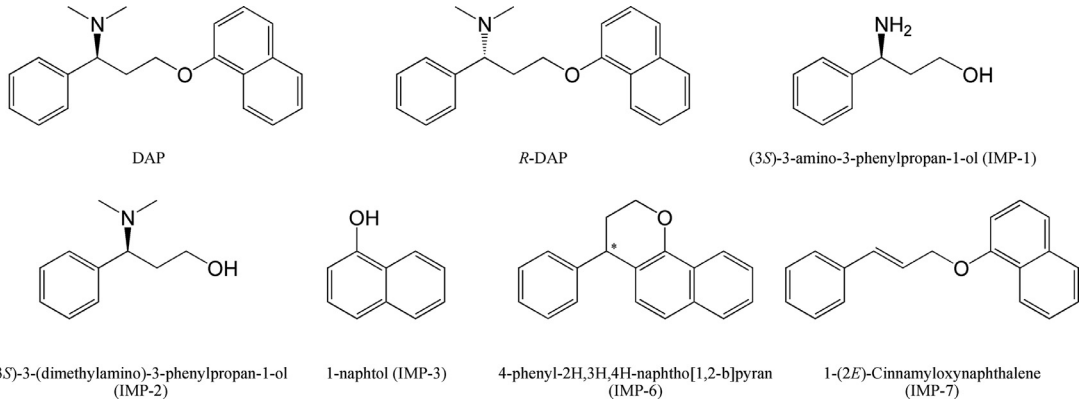

Fig. 1. Chemical name and structure of the compounds used in this study. Numbering of the organic impurities are given based on the observed elution order (see Section 3.1).

all age groups equally and due to social misconceptions, it is often under-diagnosed and under-treated. Despite its considerable impact on quality of life of men and their partners, therapeutic choice is often limited [2,3]. Although sometimes beneficial, psychological counseling and other non-pharmacological methods are often time-consuming and suffer from a lack of long-term compliance [3]. Pharmacological treatment options mostly include the use of local anesthetics, selective serotonin reuptake inhibitors (SSRIs), tramadol, phosphodiesterase type 5 inhibitors (PDE5-is) and alpha-adrenergic blockers. The introduction of SSRIs has revolutionized the pharmacotherapy of PE, particularly, the off-label use of paroxetine, sertraline, fluoxetine and citalopram for managing lifelong and acquired PE [4].

Unlike the SSRIs mentioned above, dapoxetine (DAP, (1*S*)-N,*N*-Dimethyl-3-(naphthalen-1-yloxy)−1-phenylpropan-1-amine, Fig. 1) was the first and until date, the only SSRI approved for the on-demand treatment of PE [5]. In its structure, DAP presents a single asymmetric carbon atom, it is marketed in its enantiopure form, as the *S*-enantiomer is 3.5-times more potent SSRI, than *R*-DAP [6]. Thus, several synthetic procedures were described for the asymmetric synthesis of the short-acting SSRI [7-18] and numerous enantioselective methods are also available, including chiral liquid chromatography on cellulose tris(3,5-dimethylphenylcarbamate) based columns [15,16,19], cellulose tris(4-methylbenzoate) based colum [17] and Pirkle-type (*R,R*) – Whelk O1 [11] using normal phase eluents. Capillary electrophoresis with different cyclodextrins as chiral selectors are also applied [20-23]. In a recent study, Harnisch and Scriba also developed a validated capillary electrophoretic method for the simultaneous determination of DAP enantiomers and three other related substances using a dual cyclodextrin system [24].

Counterfeit medicines represent a worldwide concern, hurting not only the pharmaceutical industry, but more importantly, patients also [25]. Due to the social burden and taboo associated with certain sexual disorders, performance enhancer drugs are among the most widely available medicines on the internet as both counterfeits or illegal herbal supplement adulterants. Although, in most cases PDE5-is get the headline, adulteration of herbal products with DAP has also been described [26].

Thus, quality control of internet-based acquisition of drugs is of utmost importance. In case of approved formulations with enantiopure drugs, in some cases there are distinct methods for assay of active substance, achiral impurities and chiral impurities. One of the main reasons for this approach is the inherently lower separation efficiency (plate number) of CSPs when compared to the typical achiral reversed-phase columns. This approach however, is extensively resource and time-consuming, which hinders their application in routine counterfeit screenings. Combined methods, which could be applied for identification, assay, chiral and achiral impurity profiling of the active substance/substances would be extremely beneficial in these cases. The bottleneck of these methods is that they need to display both enantioselectivity and chemoselectivity, allowing separation of the enantiomers and resolution of closely related compounds in the same analytic run.

Several CSPs, although proved to be versatile in enantioseparation, display insufficient chemoselectivity [27-29]. This restricts their application only for the chiral impurity profiling in routine quality control. Thus, an additional separation by a second column needs also be employed for the achiral separation and quantification of other related compounds. Bioseparations are another area where this drawback is a serious concern, especially, where simultaneous analysis of metabolites and parent compounds is attempted [30]. In various applications, a multicolumn approach, employing an additional nonenantioselective stationary phase is usually preferred to circumvent the inadequate chemoselectivity of the chiral column [31-34]. Usually, in chiral multidimensional liquid chromatography the achiral column is employed for the resolution of the analytes into less complex fractions (first dimension), followed by transferring by column switching of some or all of the fractions to the secondary, chiral column (second dimension). These approaches can be classified as linear or heart-cut two-dimensional techniques (LC-LC, when only specific fractions are transferred to the second column) and comprehensive two-dimensional chromatography (LCxLC, when all components are transferred to the second column) [35-37]. Due to the increased instrumental complexity of the multidimensional approaches, serial in-line coupling of achiral and chiral columns were also explored [27,29,38,39].

In recent years there is a growing interest in applying polysaccharide-based chiral stationary phases (CSPs) in the development of simultaneous chemo- and enantioselective liquid chromatographic methods [40–49], especially given the versatile selectivity space available in different solvents or solvent mixtures. In the present study, the applicability of polysaccharide-based CSPs in liquid chromatography was extended for the simultaneous quantification of chiral and achiral impurities of DAP, namely *R*-DAP, (3*S*)−3-amino-3-phenylpropan-1-ol (IMP-1), (3*S*)−3-(dimethylamino)−3-phenylpropan-1-ol (IMP-2), 1-naphthol (IMP-3), racemic 4-phenyl-2H,3H,4H-naphtho[1,2-*b*]pyran) (IMP-6) and 1-(2*E*)-cinnamyloxynaphthalene (IMP-7) (Fig. 1). IMP-1, IMP-2, IMP-3 and *R*-DAP are synthesis related impurities, although IMP-2 can also be present as a degradation product [50]. IMP-6 is a recently described, possible by-product of DAP synthesis, while IMP-7 is a possible degradation product of DAP. The pathways of for-

mation for IMP-6 and IMP-7 are discussed in our earlier articles [51,52].

Baseline separation of the structurally diverse set of analytes was achieved in hydroalcoholic mobile phases, using a combination of gradient elution, with flow programming. Application of the newly developed method was challenged by performing impurity profiling of pharmacy- and internet-acquired mono- and combination tablets, containing DAP.

2. Materials and methods

2.1. Materials

DAP, as a hydrochloride salt (purity > 98.0%) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Racemic DAP was purchased from Chemieliva Pharmaceutical Co., Ltd. (Chongqing, China). Impurities such as (3*S*)−3-amino-3-phenylpropan-1-ol (IMP-1) and (3*S*)−3-(dimethylamino)−3-phenylpropan-1-ol (IMP-2) were from Carbosynth (Compton, UK); 1-naphthol (IMP-3) was obtained from Merck KGaA (Darmstadt, Germany); racemic 4-phenyl-2H,3H,4H-naphtho[1,2-*b*]pyran) (IMP-6a and IMP-6b, for the first and second eluting enantiomer) and 1-(2*E*)-Cinnamyloxynaphthalene (IMP-7) were synthetized according to our previous articles [51,52]. Avanafil was ordered from Toronto Research Chemicals (North York, Ontario, Canada). Sildenafil as citrate salt was acquired from Intas Pharmaceuticals Ltd. (Ahmedabad, India), while tadalafil was from MSN Laboratories Ltd. (Hyderabad, India).

Gradient grade methanol (MeOH), ethanol (EtOH) acetonitrile (ACN) and 2-propanol (IPA) were purchased from Merck KgaA (Darmstadt, Germany). Diethylamine (DEA) was from Sigma-Aldrich Hungary (Budapest, Hungary). Ultrapure, deionized water was prepared by a Milli-Q Direct 8 Millipore system (Milford, MA, USA). All mobile phase compositions described represent volume-to-volume ratios.

All chiral columns with identical dimensions (150 × 4.6 mm, 5 μm average particle size): Lux Cellulose-1 [cellulose tris(3,5-dimethylphenylcarbamate)], Lux Cellulose-2 [cellulose tris(3–chloro-4-methylphenylcarbamate)], Lux Cellulose-3 [cellulose tris(4-methylbenzoate)], Lux Cellulose-4 [cellulose tris(4–chloro-3-methylphenylcarbamate)], Lux Amylose-1 [amylose tris(3,5-dimethylphenylcarbamate)], Lux i-Amylose-1 [amylose tris(3,5-dimethylphenylcarbamate)], immobilized and Lux Amylose-2 [amylose tris(5–chloro-2-methylphenylcarbamate)] were ordered from Phenomenex (Torrance, CA, USA).

Priligy® tablets (30 mg) (Berlin-Chemie AG) were obtained from Central Pharmacy of Semmelweis University (Budapest, Hungary). Three further products were ordered from different internet sources and had the following DAP content declared: 60 mg DAP as HCl salt (Dapoxetin HCl tablet), 60 mg DAP with 200 mg avanafil (Super Avana) as well as 60 mg DAP with 40 mg tadalafil (Super Tadarise). All of these products came without secondary packaging and leaflet (Supplementary Figure 1).

2.2. Chiral HPLC method

Method development was carried out on a JASCO HPLC system (JASCO PU-2089 Plus binary gradient pump, AS-4050 autosampler, MD-2010 Plus diode array detector and CO2065 plus column oven (JASCO International Co. Ltd., Tokyo, Japan). The software used to operate the equipment and data processing was ChromNAV. The final method was also transferred to an Agilent 1260 Infinity HPLC system (G1312B binary gradient pump, G1367E autosampler, G1315C diode array detector) (Agilent Technologies, Waldbronn, Germany), equipped with Agilent Masshunter software for data analysis.

The developed method was validated for the enantioselective assay of DAP and for the simultaneous analysis of related substances and enantiomeric purity of the active substance. In both cases, MeOH was used as the solvent for the preparation of solutions throughout the study. For the assay of DAP, the target concentration was set at 100 µg mL^{-1} and UV detection was performed at 224 nm. The final test solution of DAP used for simultaneous achiral and enantiomeric purity testing was about 5000 µg mL^{-1}. All impurity level percentages are reported to this concentration. An injection volume of 10 μL was used throughout the method.

For preparation of sample solutions, two tablets were weighted, then ground and mixed in a mortar. In a 5 mL volumetric flask, MeOH was added to an accurately weighted portion of the tablet powder corresponding to about 25 mg DAP. Then the suspension was sonicated for 30 min and centrifuged for 2 min applying 4000 rpm (Sartorius 2–16 P benchtop centrifuge, Goettingen, Germany). The clear supernatant was filtered through 0.22 μm pore size Durapore PVDF syringe filter (Millex® GV filter, Millipore, Milford, MA, USA). When necessary, sample solutions were further diluted with MeOH before the measurements. It is to be noted here, that the standard procedure of purity and content analysis usually requires the use of twenty tablets in order to ensure a representative sampling. However, in our case, since the supply of internet-acquired products was extremely low, determinations were performed using only two tablets. In the case of the pharmacy-acquired product, twenty tablets were used.

2.3. Determination of dap content by reversed-phase HPLC method

The DAP content of samples was also determined by our earlier developed HPLC method with some modification [51]. An Agilent Zorbax Eclipse Plus C18, 100 mm × 4.6 mm i.d. (3.5 μm particle size) column was applied (Agilent Waldbronn, Germany), while the mobile phase consisted of MeOH and 0.1 (v/v%) formic acid in water, using the following gradient program: 0 min 50% MeOH, 4 min 50% MeOH, 6 min 90% MeOH, 10.0 min 90% MeOH, 11 min 50% MeOH, 15 min 50% MeOH. The flow rate was set to 0.7 mL min^{-1}, while the column temperature was kept at 30 °C.

2.4. Analytical HPLC hyphenated with UV and high-resolution orbitrap mass spectrometric detections to identify compounds in counterfeit pharmaceutical preparations

A Dionex Ultimate 3000 UHPLC system (DAD-3000RS diode array detector (DAD), TCC-3000RS column thermostat, HPG-3400RS pump, SRD-3400 solvent rack degasser, WPS-3000TRS autosampler), hyphenated with a Orbitrap Q Exactive Focus Mass Spectrometer equipped with electrospray ionization (ESI) (Thermo Fischer Scientific, Waltham, MA, USA) was used for chromatographic separation and high resolution mass spectral analysis. The HPLC separations were performed on a Kinetex C18 column (75 × 3.0 mm; 3.5 μm) (Phenomenex, USA). The mobile phase consisted of 0.1% v/v formic acid in water (A) and ACN (B). Linear gradient elution was performed as follows: 0.0 min, 20% B; 12.0 min, 60% B; flow rate: 0.3 mL min^{-1}; column temperature: 25 °C; injected volume: 1.0 μL. The ESI source was operated in negative ionization mode and operation parameters were optimized automatically using the built-in software. The working parameters were as follows: spray voltage, 2500 V (-); capillary temperature 320 °C; sheath-, auxiliary- and spare-gases (N_2): 47.52, 11.25 and 2.25 arbitrary units, respectively. The resolution of the full scan was of 70,000 and the scanning range was between 100 and 1000 *m/z* units. MS/MS scans were acquired at a resolution of 35,000 in the range of 80–1000 *m/z* units, using collision energy

Table 1
Chromatographic data obtained during the preliminary study, in terms of first- and second-eluting enantiomer (t_1 and t_2, respectively), resolution (R_s) and elution order for the chromatographic systems, where enantiorecognition was observed.

Column type	Mobile phase	t_1 (min)	t_2 (min)	R_s	Elution order
Lux Cellulose-1	MeOH:DEA 100:0.1 (v/v)	7.75	8.23	0.92	*S*<*R*
Lux Cellulose-1	ACN:DEA 100:0.1 (v/v)	5.86	6.04	1.07	*R*<*S*
Lux Cellulose-3	MeOH:DEA 100:0.1 (v/v)	10.04	11.29	2.42	*S*<*R*

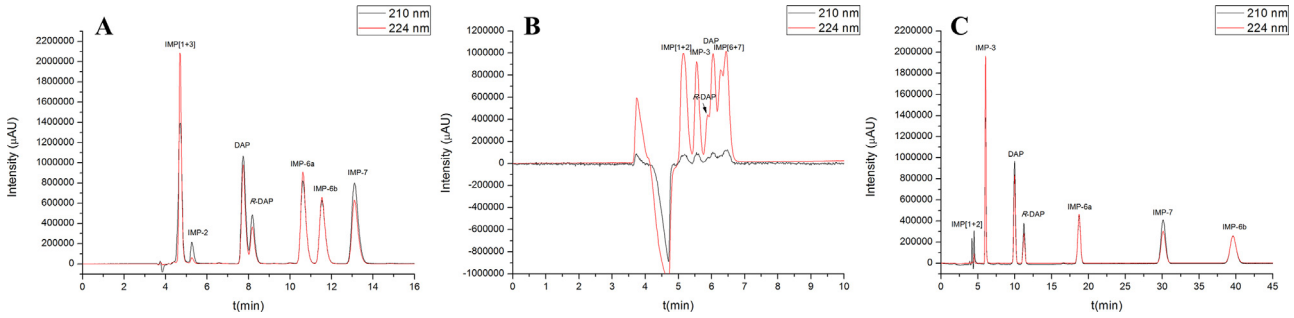

Fig. 2. Separation of DAP and its chiral and achiral impurities using (A) Lux Cellulose-1 with MeOH:DEA 100:0.1 (v/v), (B) Lux Cellulose-1 with ACN:DEA 100:0.1 (v/v), (C) Lux Cellulose-3 with MeOH:DEA 100:0.1 (v/v). All columns had identical dimensions: 150 mm x 4.6 mm, 5 µm. Other chromatographic parameters: flow rate: 0.5 mL min^{-1}, column temperature: 20 °C, 10 µL injection volume, detection at 210 and 224 nm.

of 10, 20, 30 and 45 eV. DAD spectra were also recorded between 250 and 600 nm.

3. Results and discussion

3.1. Method development

As a first step in method development, the chiral separation of DAP enantiomers was attempted, as the most critical part. Seven different polysaccharide-based CSPs, including amylose-based Lux Amylose-1, Lux i-Amylose-1 and Lux Amylose-2, as well as cellulose-based Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3 and Lux Cellulose-4 were tested in polar organic mode using 0.1% (v/v) DEA in MeOH, IPA or ACN as mobile phases, with 0.5 mL min^{-1} flow rate at 20 °C. During the preliminary experiments, from the twenty-one separation systems employed, enantiorecognition was observed only in three cases (Table 1).

Apart from enantioseparation, chemoselectivity of the method was further tested on all three systems, by injecting all of the studied analytes (Fig. 2).

Using the ACN-based mobile phase on the Lux Cellulose-1 column, containing cellulose tris(3,5-dimethylphenylcarbamate) as a chiral selector, poor retention with inadequate chemoselectivity could be observed. Applying the MeOH-based mobile phase on the same column increased retention and improved chemoselectivity of the system. Interestingly, using MeOH instead of ACN, changed the enantiomer elution order to the more favorable distomer-first elution.

The best results both in terms of enantio– and chemoselectivity were obtained using the cellulose tris(4-methylbenzoate)-based Lux Cellulose-3 column with MeOH:DEA 100:0.1 as mobile phase. In spite of the eutomer-first elution order, this system provided adequate peak spacing not only for the enantiomers, but for the other closely related compounds also. Thus, this combination was employed for additional method optimization.

Further method optimization aimed to:

1 increase the capacity factor of IMP-1 and IMP-2
2 increase the resolution between DAP enantiomers
3 shorten the analysis time

Table 2A
Mobile phase gradient of the optimized method.

Time (min)	EtOH:DEA 100:0.1 (v/v)	H_2O
0	35	65
5	35	65
8	100	0
30	100	0

Table 2B
Flow rate gradient of the optimized method.

Time (min)	Flow rate (mL min^{-1})
0	0.5
8	0.5
10	0.8
16	0.8
18	1.0
30	1.0

Due to sample overload and subsequent peak tailing, a higher enantioresolution is crucial in cases where the main chromatographic peak elutes first and low impurity levels are expected. In order to increase enantioresolution, first, MeOH was replaced with EtOH in the mobile phase This resulted in an increase in enantioselectivity (R_s of enantiomers increased to 7.15) (Supplementary Figure 2), but also further decrease in capacity factor of IMP-1 and IMP-2.

To circumvent the problem, different approaches were tested, such as varying DEA concentrations (from EtOH:DEA 100:0.05 to EtOH:DEA 100:0.25), using mixtures of DEA with formic acid (FA) or acetic acid (AA) (EtOH:DEA:FA 100:0.1:0.1, EtOH:DEA:AA 100:0.1:0.1) and using EtOH:IPA mixtures (EtOH:IPA:DEA 95:5:0.1, EtOH:IPA:DEA 90:10:0.1, EtOH:IPA:DEA 80:20:0.1) without success.

In a typical reverse-phase behavior, adding water (20–65% v/v) in the EtOH-based mobile phase, increased IMP-1 retention. However, in these conditions the total analysis time was above 120 min, therefore different gradient methods were also tested, to reduce it. In order to counterbalance the increased total analysis time, higher column temperature (40 °C) and gradient elution (Table 2A), combined with flow- programming (Table 2B) was applied. The

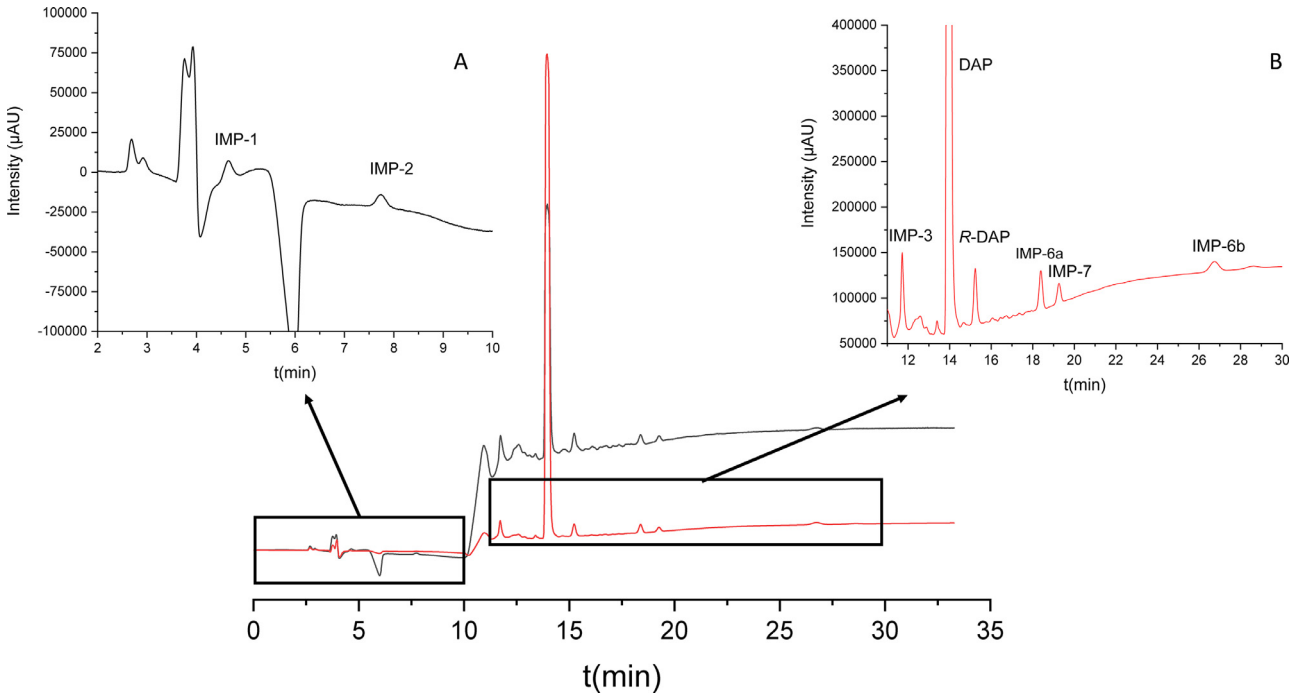

Fig. 3. Chromatograms obtained with the final method for a standard solution containing 5000 µg mL^{-1} DAP spiked with 5 µg mL^{-1} IMP-1, IMP-2 and IMP-7, 2.5 µg mL^{-1} IMP-3, *R*-DAP, IMP-6a and IMP-6b. Concentrations corresponded to the respective LOQ values of analytes, except IMP-6b (LOD value). Detection was performed at 210 nm (for IMP-1 and IMP-2) and 224 nm for all other analytes. Column: Lux Cellulose-3, 150 × 4.6 mm, 5 µm, column temperature: 40 °C, 10 µL injection volume. Mobile phase, gradient profile and flow programming are given in Table 2.

selected conditions resulted in baseline separation of all analytes within 30 min (Fig. 3).

3.2. Method validation

Method validation for simultaneous achiral and enantiomeric purity analysis was performed according to International Council for Harmonization guideline Q2 (R1) for all analytes, with respect to sensitivity, linearity, accuracy and precision [53]. Detection wavelengths were 210 nm for IMP-1 and IMP-2 and 224 nm for all of the other analytes. Sensitivity of the method was evaluated in terms of limit of detection (LOD) and limit of quantification (LOQ). These were estimated based on signal-to-noise ratio of 3:1 (LOD) and 10:1 (LOQ). Determination of impurities was validated in the range of 0.1–1% for *S*- and *R*-DAP, IMP-1, IMP-2, IMP-3, IMP-7 and 0.05–0.5% for IMP-6a and 0.16–0.5% for IMP-6b, with regard to a target concentration of 5000 µg mL^{-1} of DAP. Validation data is summarized in Table 3. Because of low peak efficiencies recorded for the last eluting IMP-6b, LOQ values do not meet the reporting threshold stipulations (0.1% impurity) of current ICH Q3B(R2) guideline [54]. However, it should be noted that IMP-6 is a racemate, and low levels of IMP-6b could be monitored indirectly by quantification of IMP-6a. This is also supported by the ratio of the determined relative response factors (RRFs), which is approximately 1 (Supplementary Table 1, see below).

Linearity of the method was established at eight concentration levels within the indicated range, for all impurities. Calibration plots, constructed by plotting peak areas against corresponding impurity concentrations (expressed in µg mL^{-1}) indicated a linear relationship, with correlation coefficients higher than 0.998 in all cases, while 95% confidence intervals of the y-intercepts included zero and residuals indicated random distributions. RRFs were calculated as the ratio of the slope of the calibration curve of the given impurity at its monitoring wavelength and the slope of the calibration curve of DAP at 224 nm. Results are compiled in Supplementary Table 1.

Accuracy and precision were analyzed by performing intraday and interday (intermediate precision) evaluations by five replicate injections of three concentration levels of all impurities, covering the linearity range. Injections were performed on the same day and on two consecutive days.

For all impurities, accuracy (expressed in average recovery%) ranged from 96.15% to 104.15%. Intraday precision, (expressed as RSD%) was between 0.15% - 2.95%, while RSD for intermediate precision was below 2.80%. Based on the results obtained during validation, the method proved to be sensitive, linear, accurate and precise for the determination of the selected impurities in DAP.

Given the complexity of the developed method (gradient and flow-programming), an inter-instrumental method transfer was also initiated. Using an Agilent 1260 Infinity HPLC system the main objective was to observe whether instrumental changes would hinder the applicability of the method. The obtained chromatogram is depicted in Supplementary Figure 3, showing that the method was repeatable and inter-instrumental variations did not affect method reliability. Although slight differences in retention times were observed, baseline separations of critical peak pairs (DAP – *R*-DAP and IMP-6a – IMP 7) were maintained. Comparative retention times of the analytes and the obtained resolution values are presented in Supplementary Table 2.

The method was also validated for the enantioselective assay of DAP. In this case, the target sample concentration was set at 100 µg mL^{-1} and linearity, accuracy, precision (both intra- and interday precision) were assessed at 224 nm. Based on the obtained results (Supplementary Table 3), the method proved to be adequate for the quantitative measurement of DAP.

3.3. Method application

Purchasing prescription-only pharmaceuticals from unauthorized sources over the internet, increases the risk of receiving substandard or counterfeit medicines. These can include one or more of the following possible scenarios: (a) pharmaceuticals without

Table 3
Summary of data obtained during method validation for the simultaneous determination of related substances and enantiomeric purity.

Parameter	Level	IMP-1	IMP-2	IMP-3	DAP	*R*-DAP	IMP-6a	IMP-7	IMP-6b
Range (µg/mL)		5 – 50	5 – 50	5– 50	5 – 50	5 – 50	2.5 – 25	5–50	8 – 25
Range (%)		0.1% - 1%	0.1% - 1%	0.1% - 1%	0.1%–1%	0.1% - 1%	0.05% - 0.5%	0.1%–1%	0.16%–0.5%
r^2		0.9995	0.9981	0.9984	0.9993	0.9990	0.9981	0.9990	0.9983
LOD (µg/mL)		1.5	1.5	0.75	0.75	0.75	0.75	1.5	2.5
LOQ (µg/mL)		5	5	2.5	2.5	2.5	2.5	5	8.0
Accuracy (%)	I*	97.90 ± 0.50	100.32 ± 1.01	99.79 ± 0.33	100.23±0.54	102.14 ± 0.72	104.15 ± 1.01	96.15± 1.06	96.17± 1.58
	II**	96.52 ± 1.02	99.55 ± 1.21	100.65 ± 0.59	99.85±0.21	98.11 ± 0.56	98.05 ± 0.54	97.03± 2.45	101.11± 3.15
	III***	100.29 ± 0.42	101.51 ± 0.68	100.51 ± 0.35	99.54±0.49	99.02 ± 0.35	98.62 ± 0.69	100.5 ± 0.12	103.15± 1.89
Intraday precision (RSD%)	I*	2.95	1.05	1.75	1.24	1.89	1.01	1.54	2.55
	II**	0.45	0.84	1.01	0.25	1.02	0.89	0.45	1.89
	III***	0.74	0.15	0.65	0.85	0.49	0.75	0.46	1.75
Intermediate precision (inter-day) (RSD%)	I*	1.12	1.65	2.56	1.29	1.45	1.15	2.15	2.78
	II**	0.78	0.45	0.99	0.34	1.12	0.54	2.45	2.65
	III***	0.54	0.52	0.71	0.90	0.52	0.24	1.01	1.97

* Level I: IMP-1, IMP-2, IMP-3, DAP *R*-DAP, IMP-7: 5 µg mL^{-1}; IMP-6a: 2.5 µg mL^{-1}, IMP-6b: 10 µg mL^{-1}.
** Level I: IMP-1, IMP-2, IMP-3, DAP, *R*-DAP, IMP-7: 10 µg mL^{-1}; IMP-6a: 5 µg mL^{-1}, IMP-6b: 15 µg mL^{-1}.
*** Level I: IMP-1, IMP-2, IMP-3, DAP *R*-DAP, IMP-7: 40 µg mL^{-1}; IMP-6a: 20 µg mL^{-1}, IMP-6b: 20 µg mL^{-1}.

Table 4
Summary of results obtained during testing the acquired DAP-containing products.

Product	Source	Labeled	Identified components	DAP content (mg)	*R*-DAP
Priligy	Pharmacy	30 mg DAP	DAP	29.93±0.32	<LOQ
Dapoxetine HCl tablet	Internet	60 mg DAP	DAP	13.49±0.39	5.39±0.15
Super Avana	Internet	60 mg DAP + 200 mg avanafil	DAP + avanafil + sildenafil	30.14±0.73	30.08±0.87
Super Tadarise	Internet	60 mg DAP + 40 mg tadalafil	DAP + tadalafil	59.01±1.10*	29.89±0.45

* Obtained using an earlier developed achiral method (results expressed as racemic DAP). Tadalafil and DAP were not baseline resolved.

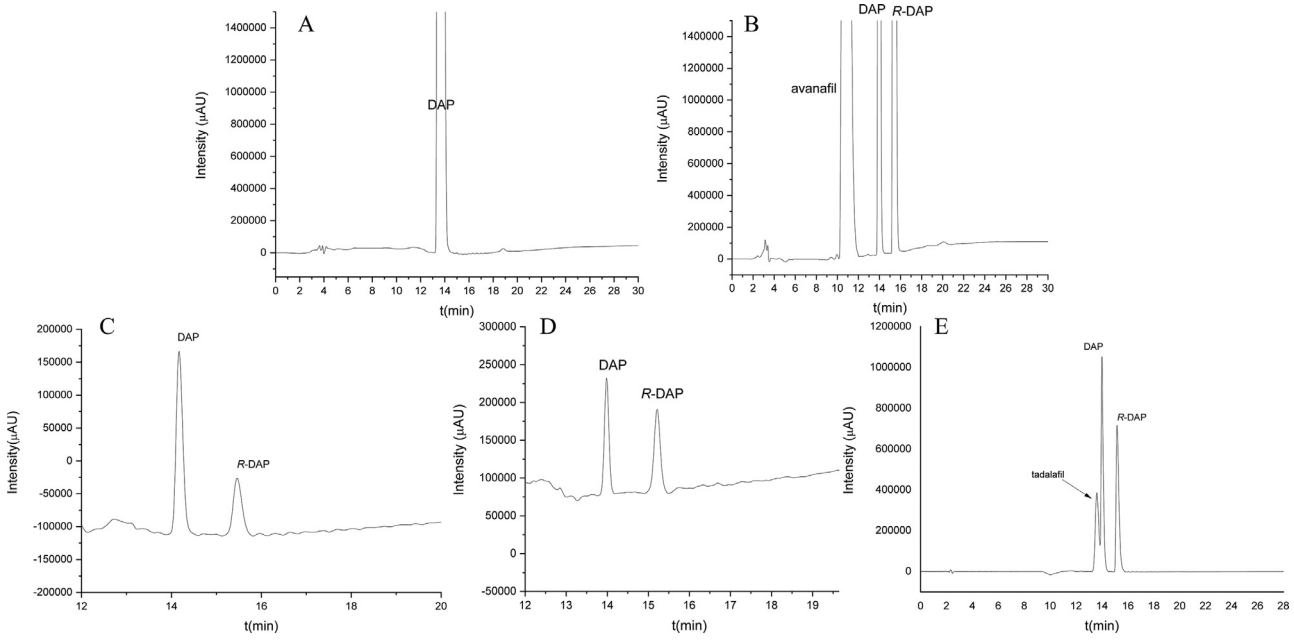

Fig. 4. Chromatograms of a (A) sample solution of Priligy® tablet, (B) sample solution of Super Avana Tablet. Determination of enantiomeric ratio of DAP and *R*-DAP. Diluted sample solution of (C) Dapoxetine HCl tablet, (D) Super Avana tablet, (E) Super Tadarise tablet. Experimental conditions: Lux Cellulose-3, 150 × 4.6 mm, 5 µm, column temperature: 40 °C, 10 µL injection volume. DAP concentration: aprox. 5000 µg/mL (A, B), aprox. 100 µg/mL (C,D) and aprox. 500 µg/mL (E). Mobile phase, gradient profile and flow programming are given in Table 2. Detection: 224 nm.

active ingredient; (b) contaminated pharmaceuticals with no active ingredient (API), (c) wrong API in counterfeit drug; (c) pharmaceuticals with wrong concentration or wrong dose of API; (d) pharmaceuticals which contain undeclared API [25,55,56]

Taking into account the abovementioned considerations, the first step in analyzing the acquired pharmaceuticals was to utilize an online HPLC-ESI-Orbitrap-HRMS method to identify DAP and other possible components from the products (Table 4). As expected, in the approved, originator product, only DAP was detected. In two of the three internet-acquired products, only the declared active ingredients were detected (DAP in Dapoxetine HCl tablets, while DAP and tadalafil were detected in Super Tadarise). A pecu-

liar case was that of the product Super Avana, in which apart from DAP and avanafil, sildenafil was also detected (Supplementary Figure 4).

DAP content was determined in all products based on the validated enantioselective assay. However, when analyzing the combination product, Super Tadarise, baseline resolution between tadalafil and DAP was not achieved. Thus, our earlier developed achiral HPLC method was applied for the estimation of racemic DAP. Based on the obtained results (Table 4), Priligy and two other products acquired from the internet – Super Avana and Super Tadarise – present the declared amount of DAP, however for Dapoxetine HCl tablet, only 13.49±0.39 mg active substance was obtained

A surprising result was observed when the impurity-profile of the purchased products was further investigated (Fig. 4). As it was expected, based on the former CE study [24] in Priligy® all related substances and *R*-DAP concentration were below the LOD. However, in the products ordered from internet racemic DAP and/or high concentration of *R*-DAP was detected. Super Avana and Super Tadarise contain the racemic mixture, while in Dapoxetine HCl tablet a scalemic mixture with the enantiomeric ratio of 1:0.4 is present.

4. Concluding remarks

A single-run HPLC method using Lux Cellulose-3 column in reversed-phase mode was developed for the simultaneous determination of related substancesIMP-1, IMP-2, IMP-3, IMP-6 and IMP-7 as related substances and enantiomeric impurity *R*-DAP in DAP. The method was validated according to the International Council for Harmonization guideline Q2(R1) and proved to be precise and accurate for determination of at least 0.1% or below for all impurities in DAP samples. Application of the method was tested on a commercial tablet and products from internet and could be applied in an industrial environment for simultaneous quantification of chemical and chiral related substances of DAP in one run to save time and costs.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of competing interest

The authors have declared no conflict of interest

CRediT authorship contribution statement

Gergő Tóth: Methodology, Investigation, Funding acquisition, Writing - original draft. **Erzsébet Fogarasi:** Methodology, Investigation, Validation, Writing - original draft. **Ágnes Bartalis-Fábián:** Investigation, Validation. **Mohammadhassan Foroughbakhshfasaei:** Investigation, Validation. **Imre Boldizsár:** Investigation, Funding acquisition, Writing - review & editing. **András Darcsi:** Investigation, Resources, Writing - review & editing. **Szilvia Lohner:** Investigation, Writing - review & editing. **Gerhard K.E. Scriba:** Resources, Writing - review & editing. **Zoltán-István Szabó:** Conceptualization, Methodology, Project administration, Writing - review & editing, Conceptualization, Methodology, Project administration, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461388.

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