Article

Simultaneous Determination of Tramadol and *O*-Desmethyltramadol in Human Plasma Using HPLC–DAD

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A sensitive and accurate method for the extraction and quantification of tramadol (T) and its active metabolite, *O*-desmethyltramadol (ODT) in human plasma with high-performance liquid chromatography–diode array detection was developed and validated. The analytes were extracted from plasma samples by *tert*-butylmethyl ether in the presence of ammonium hydroxide as alkaline medium and back extraction with 1.0 M hydrochloric acid. Propranolol was used as internal standard. The extraction efficiencies of T and ODT were 83.51 and 78.72%, respectively. The calibration curves were linear ($r^2 > 0.99$) in the concentration range of 250–2000 ng/mL for T and ODT. Limits of detection and quantification were 125 and 250 ng/mL for both analytes. Intra- and interassay precision for T and ODT were ranged from 1.89 to 10.91% and 2.16 to 5.85%, respectively. Intra- and interassay accuracy for T and ODT were ranged from −13.07 to 4.99% and −2.03 to −6.98%, respectively. The method was successfully applied to quantify T and ODT from authentic plasma samples received from Hospital Sohag University. The method was completely validated and can be of interest to clinical and forensic laboratories.

Introduction

Tramadol hydrochloride (T), ($\pm$)-*trans*-2-[(dimethyl-amino) methyl]-1-(3-methoxyphenyl)cyclohexanol, is a centrally acting analgesic agent used in the treatment of moderate to severe pain and as an alternative to opiates (1). In humans, the drug undergoes significant metabolism by cytochrome P4502D6 to *O*-desmethyltramadol (ODT) and *N*-desmethyltramadol being the main Phase 1 metabolites. These primary metabolites may be further metabolized to three additional minor metabolites, namely *N*, *N*-didesmethyltramadol, *N*, *N*, *O*-tridesmethyltramadol and *N*, *O*-desmethyltramadol. All metabolites are further conjugated with glucuronic acid and sulfate before excretion in urine (2). The metabolite ODT is pharmacologically active and is mainly responsible for the analgesic efficacy of tramadol (T) (3).

The methods described for the determination of T in biological samples involve gas chromatography (GC) with nitrogen–phosphorous detection (4), flame ionization detection (5) or mass spectrometry (MS) (6–15). Methods involving liquid chromatography (LC) with ultraviolet (16–19), fluorescence (20–31), electrochemical (32), diode array (33–35) or MS detection (36–43) were also reported.

Up to date, high-performance liquid chromatography–diode array detection (HPLC–DAD) is still one of the analytical tools in forensic and clinical laboratories for the analysis of most common drugs in biological matrices (44–46), due to full scan

UV spectra for common drugs and lower cost than GC–MS and LC–MS/MS. Simultaneous quantification of the parent drug and one or more metabolites give confident and reliable date. A HPLC–DAD method for quantification of T and its main metabolite ODT in human plasma was not described so far. Therefore the purpose of this study was to develop and validate analytical procedure for simultaneous extraction and quantification of T and ODT in human plasma, and to use this method in routine analysis of T and ODT in plasma samples at Clinical Toxicology Laboratory (CTL) of Sohag University, Egypt. Additionally, the method is successfully tested for screening and quantification of T and ODT by external quality control of the College of American Pathologists (CAP).

Experimental

Instrumentation and conditions

The HPLC instrument (Agilent, USA) consisted of an Agilent technologies 1200 Series quaternary pump combined with an Agilent 1200 series photodiode array detector (USA), an Agilent 1200 series vacuum degasser (USA) and an Agilent autosampler injector. Chromatographic separation was performed on a Zorbax SB-C8 (250 × 4.6 mm, 5 μm) column (USA) maintained at 25°C. The mobile phase consisted of acetonitrile:buffer (0.01 M) potassium dihydrogenphosphate (50 : 50, v/v) with the addition of 0.1% triethylamine adjusted to pH 5.5 with 0.1 M sodium hydroxide) at a flow rate of 1.0 mL/min. The detector was set to scan from 200 to 800 nm and had a discrete channel set at 218 nm, which was the wavelength used for quantification.

Chemicals and reagents

Tramadol hydrochloride (Figure 1a) (purity >99%), propranolol hydrochloride (purity 98%) (Figure 1b) and diethyl ether (99% GC) were purchased from Fluka Chemie GmbH, Buchs (Switzerland). *O*-Desmethyl-*cis*-tramadol hydrochloride (1.0 mg/mL) (99.4%) was supplied by Cerilliant and purchased from LGC Promochem (UK). Methyl-*tert*-butyl ether (MTBE) (98%), acetonitrile (99.9%), methanol (99.9%), triethylamine (99%) ammonium hydroxide (33%) and potassium dihydrogen phosphate (98–100.5%) were purchased from Sigma-Aldrich (Germany). Sodium hydroxide (99%) was purchased from Egyptian Co. for Chemicals and Pharmaceuticals. Phosphate buffer (0.01 M) was prepared by dissolving 1.36 g (0.01 mol) of potassium dihydrogen phosphate in 1 L deionized water.
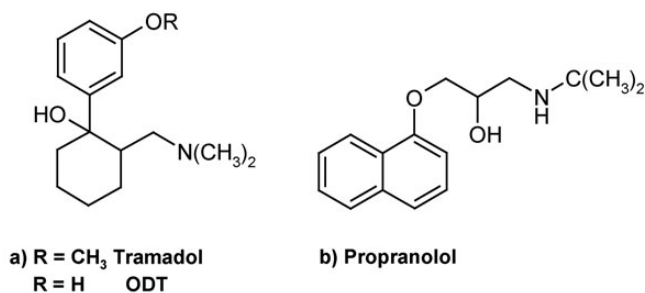
Figure 1. Structures of tramadol (T), O-desmethyltramadol (ODT) and propranolol (IS).

Calibrators and quality controls

A stock solution of T and propranolol used as internal standard (IS) were prepared at a concentration of 1 mg/mL in methanol and kept stored at $-20°C$. Intermediate standards at concentration of 100 μg/mL for each analyte were prepared in methanol by diluting from 1.0 mg/mL stock standards. Different stock standards were used to prepare quality control samples (QCs) at the same concentrations. Working calibrators (250, 500, 750, 1000, 1500 and 2000 ng/mL) of T and ODT were made by a serial dilution of the intermediate solution with drug free human plasma. QCs were prepared from a separate stock solution at concentrations of 450, 900 and 1800 ng/mL. A working standard solution of 5.00 μg/mL propranolol (IS) was prepared by diluting propranolol stock solution with distilled water.

Extraction procedure

To 10-mL polypropylene tubes added 1.0 mL of plasma, 75 μL of 5 μg/mL propranolol (IS), 100 μL of conc. ammonium hydroxide (33%) and 6.0 mL of MTBE. The tubes were then mixed at the rate of 40 rpm for 20-min and centrifuged at 3200 rpm for 5-min. The organic layer was transferred to 10-mL polypropylene tube containing 0.5 mL of 1.0 M hydrochloric acid. The tubes were then vortex mixed for 5-min and centrifuged at 3200 rpm for 5-min. The organic layer was discarded. To the remaining aqueous solution, 150 μL of conc. ammonium hydroxide and 2.0 mL of MTBE were added. The tubes were then centrifuged at 3200 rpm for 5-min. The organic layer was transferred to 5-mL glass tubes and evaporated to dryness. The dried extracts were reconstituted in 200 μL acetonitrile, vortex mixed for 30-s and 100 μL was injected into the HPLC system.

Validation methodology

A thorough and complete method validation of T and ODT in human plasma was done following the USFDA guidelines (47). Specificity, linearity, limits of detection (LOD) and quantification (LOQ), intra- and interassay precision, accuracy, extraction efficiency and stability were investigated to evaluate the method integrity.

To evaluate the method *specificity*, six different blank (no analyte or IS) and negative (IS added) plasma specimens were evaluated for co-eluting chromatographic peaks that might interfere with detection of analytes or IS. In addition, potential interferences from commonly encountered basic drugs were added to the drug free plasma specimens and subjected to the same extraction and analysis procedures. The following drugs were analyzed using the described procedures at a concentration of 1000 ng/mL: methamphetamine, meperidine, morphine, codeine, diphenhydramine, ketamine and venlafaxine,

"Linearity" of the method was investigated by evaluation of the regression line and expresses by coefficient of determination (r^2). Linearity was achieved with a minimal r^2 of 0.99. Calibration curves ($n = 4$) were prepared by spiking blank plasma with corresponding analytical working solutions to obtain calibration concentrations within 250–2000 ng/mL. Negative QCs were analyzed after each linearity sample to evaluate potential carry-over.

Sensitivity was evaluated by determining limits of detection (LOD) and quantification (LOQ) for T and ODT. The LOD was defined as signal-to-noise ratio ≥ 3 (determined by peak height) with satisfactory chromatography (peak shape and resolution) and acceptable retention time. The LOQ was defined as signal-to-noise ratio ≥ 10.

Intra- and inter-assay accuracy and precision for each analyte were determined at LQC, MQC and HQC. Intra-assay data were assessed by comparing data from within one run ($n = 6$) and interassay data were determined between three separate runs ($n = 18$). The accuracy and precision was calculated and expressed in terms of % bias and percent relative standard deviation (%RSD), respectively. Accuracy was calculated by comparing mean calculated concentrations of T and ODT in validation samples to target concentrations. Precision, was determined by calculating the percent ratio of the standard deviation divided by the calculated mean concentration times 100.

"The extraction efficiency" (%) for each analyte was determined at LQC, MQC and HQC ($n = 4$). Extraction recoveries of T, ODT and IS were calculated by comparing peak areas obtained from processed quality control plasma samples with those achieved after direct injections of standard solutions at the same concentrations.

"Analyte stability" was evaluated at LQC and MQC ($n = 4$). Stability of T and ODT in plasma samples was tested for 48 h at room temperature and $4°C$. Stability of T and ODT after extraction at ambient temperature was evaluated over 24 h. The extracted QCs were analyzed immediately after extraction along with calibrators and re-injected and analyzed after 24 h. All samples were quantified using initial calibration curves.

Application of the method

Authentic plasma samples were received from Hospital Sohag University for analysis of tramdaol from patients and drug abuser. Plasma samples were collected and stored at $-20°C$ until analysis.

Results

Optimization of the chromatographic conditions

Several parameters such as detector wavelength, mobile phase composition, pH and flow rate were studied to determine the one(s) that give the best separation. With regard to UV spectra, 218 nm was selected for the UV detection, that exhibited the best peak height for both analytes and IS. The percentage of the mobile phase of organic solvents were varied using different combinations of methanol or acetonitrile−(0.01 M) phosphate

buffer (30:70, 35:65, 40:60, 45:55, 50:50 and 60:40). The aceto-nitrile−phosphate buffer (50:50, v/v) was selected as mobile phase for faster elution, peak shape and least band tailing. The pH of the mobile phase was varied between a range of 2.0 and 7.0, where pH 6.0 or above produced band tailing and prolonged total run time. However, pH ≤ 3 the retention was dropped, hence affecting the resolution. Therefore, pH 5.0 was selected for the mobile phase, giving the best peak height count with the least band tailing for all compounds.

Average retention times for T, ODT and IS were determined as 3.42 ± 0.01, 4.75 ± 0.01 and 6.00 ± 0.01 min, respectively (total run time of 10.00 min).

To isolate T and ODT from plasma sample, direct liquid−liquid extraction (LLE) or followed with back extraction into the hydrochloric acid were tested using different organic solvent such as MTBE, diethyl ether, ethyl acetate and hexane−ethyl acetate (4:1). A clean chromatogram and very high extraction efficiencies for all analytes were obtained with MTBE.

Specificity, sensitivity and linearity

Six different blank plasma specimens were analyzed to evaluate chromatographic interference. The chromatograms show excellent peak shape for both the analytes and IS. No endogenous interferences were found at the retention times of T, ODT and IS in the blank plasma. Figure 2 demonstrates the chromatograms of blank plasma fortified with IS and fortified with T and ODT. The peaks purity of T, ODT and IS were also investigated by photodiode array detector and found to be pure (peak purity ≥99). The LOD and LOQ for T and ODT were 100 and 250 ng/mL, respectively.

The calibration curves for T and ODT were linear over the dynamic concentration range of 250−2000 ng/mL within four regression curves. Calculated concentrations of each calibrator were compared with target and were within ± 20%. Negative QCs were analyzed after each linearity sample to evaluate potential carry-over. No detectable carry-over occurred following spiked samples at the linearity limit (2000 ng/mL). The mean regression equations for T and ODT were $y = 0.00073x − 0.02635$, $r^2 = 0.991$ and $y = 0.00068x − 0.04680$, $r^2 = 0.994$, respectively.

Precision, accuracy, recovery and stability

Precision and accuracy of the method were evaluated at three concentrations (LQC, MQC, HQC) over the linear dynamic range are presented in Tables I and II. Six replicates at each concentration were assayed to determine intra-assay accuracy and precision. Intra- and interassay precision for T were ranged from 2.29 to 8.40% and 2.16 to 5.85%, respectively. Intra- and interassay accuracy for T were ranged from −1.22 to −13.07% and −4.16 to −6.98%, respectively. Intra- and interassay precision for ODT were ranged from 1.89 to 10.91% and 2.21 to 5.15%, respectively. Intra- and interassay accuracy for ODT were ranged from −7.62 to 4.99% and −2.03 to −4.79%, respectively.

The extraction efficiencies of T, ODT and IS were 83.51, 78.72 and 87.84%, respectively. Stability of T and ODT in plasma samples at MQC was evaluated at room temperature and 4°C. Analyte concentrations in short-term stability experiments were <13% of the target concentration Table III. Stability of analyte

after extraction also was examined after 24 h, both analytes were stable, differing from samples injected immediately by <10%.

Application of the method

The applicability of this method has been demonstrated by the quantification of the T and its main metabolite, ODT in human plasma samples collected from patients at Hospital Sohag University. Approximately, all samples including the calibration, QC and authentic samples were run and analyzed successfully. The precision and accuracy for calibration and QC samples were well within the acceptable limits.

Discussion

This manuscript describes an analytical procedure for the simultaneous quantification of T and its main active metabolite ODT in human plasma by HPLC−DAD following LLE. The presented extraction procedure offers a rapid way to isolate T and ODT from the plasma matrix. Whereas extraction with MTBE in the presence of ammonium hydroxide and subsequent purification by back-extraction into hydrochloric acid results clean chromatograms and very high extraction efficiencies for all analytes.

High sensitivity and small sample volume are continuous goals. Higher sensitivity increases windows of detection and permits a more accurate determination of pharmacokinetic parameters. Kucuk et al. (34), reported plasma LOD and LOQ of 200 and 350 ng/mL for T with HPLC−DAD following LLE. The present method provides good sensitivity with LOD and LOQ of 100 and 250 ng/mL for T and ODT, respectively. The salient features of some selected methods for simultaneous determination of T and ODT by HPLC−DAD are complied in Table IV.

Compared with other published HPLC methods (17, 23−28, 31), the HPLC−DAD method confers the advantage of identifying the analyte by both retention time and UV spectrum. Other hand, the presented method using low-cost and high availability instrument for quantification of T and ODT than other methods (37, 38, 41, 43),

The validated method was successfully applied to quantify T and ODT from unknown sample sent by the CAP as proficiency testing scheme to CTL of Sohag University. The concentrations of T and ODT after analysis were 1412.6 and 320 ng/mL, respectively. The concentrations of T and ODT which given by CAP after analysis were 1200 and 300 ng/mL (± 20%), respectively. Figure 3 shows the HPLC chromatograms for the analysis of the unknown sample provided by CAP and spiked T and ODT at concentration of 450 ng/mL (LQC).

Moreover, the presented method is currently used in routine analytical toxicological analysis for detection and quantification of T and ODT at Hospital Sohag University.

Conclusions

A validated method for the detection and quantification of T and ODT in human plasma by HPLC−DAD is presented. The method has suitable linearity, sensitivity, accuracy and precision with high analyte recoveries. The method was successfully applied to analysis of authentic samples provided by Sohag Hospital University and external quality control assays by the CAP. This
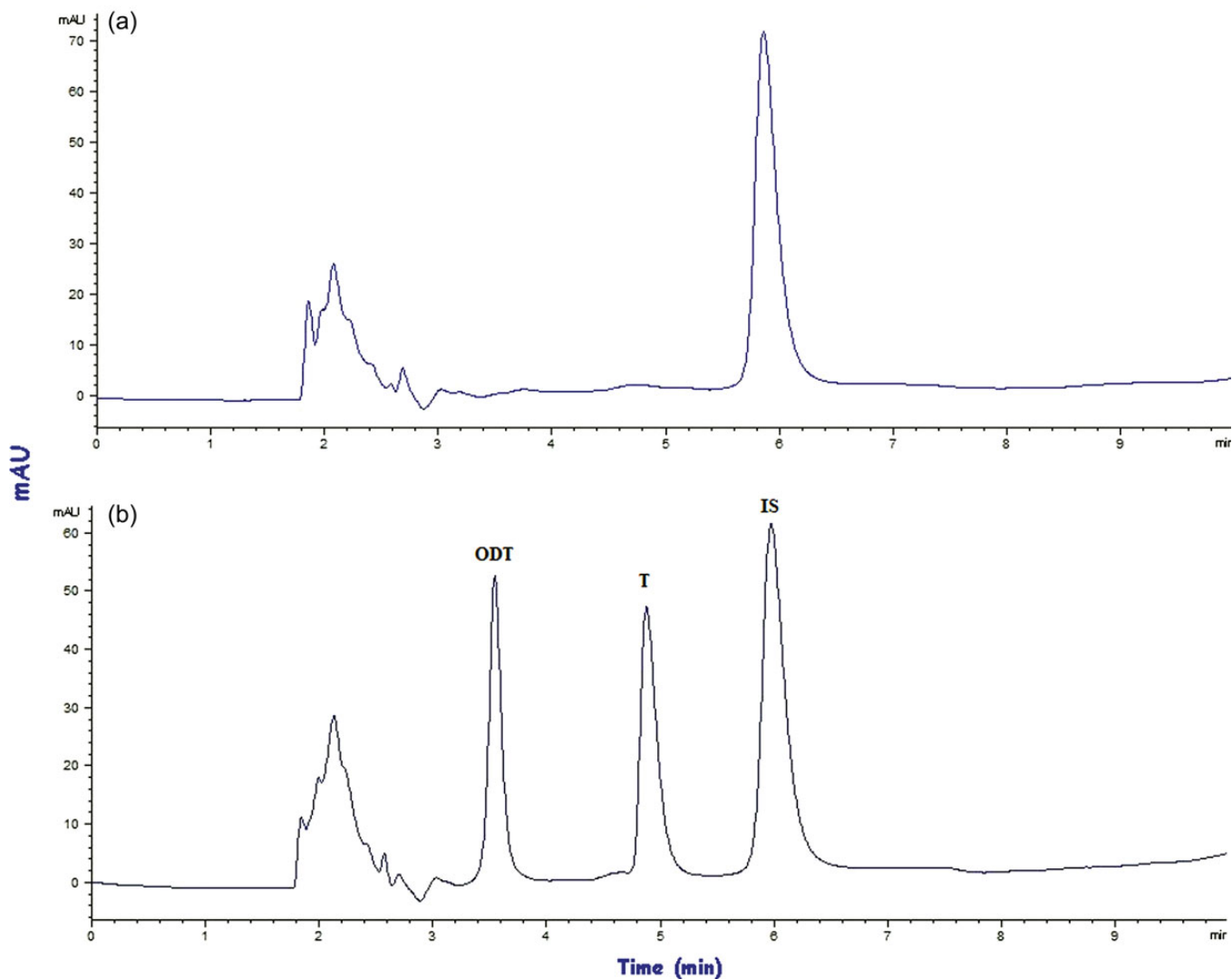
Figure 2. HPLC chromatograms of analysis of human blank plasma with (a) IS and (b) spiked with T, ODT and IS.

Table I
Intra- and Interassay Precision and Accuracy for Tramadol in Human Plasma

Nominal concentration (ng/mL)	Found concentration (ng/mL)	Precision (% RSD)	Accuracy (% Bias)
Intra-assay ($n = 6$)			
LQC (450)			
Day 1	426.05	4.15	− 5.32
Day 2	438.47	2.84	− 2.56
Day 3	391.21	2.29	− 13.07
MQC (900)			
Day 1	863.15	7.38	− 4.09
Day 2	889.01	6.16	− 1.22
Day 3	810.04	7.51	− 10.00
HQC (1800)			
Day 1	1716.94	8.40	− 4.61
Day 2	1692.62	3.20	− 5.97
Day 3	1765.67	6.54	− 1.91
Interassay ($n = 18$)			
LQC	418.57	5.85	− 6.98
MQC	854.07	4.71	− 5.10
HQC	1725.07	2.16	− 4.16

RSD, relative standard deviation.

Table II
Intra- and Interassay Precision and Accuracy for *O*-Desmethyl Tramadol in Human Plasma

| Nominal concentration (ng/mL)	Found concentration (ng/mL)	Precision (% RSD)	Accuracy (% Bias)
Intra-assay ($n = 6$)			
LQC (450)			
Day 1	451.91	5.43	0.42
Day 2	438.66	3.07	− 2.52
Day 3	419.97	1.89	− 6.67
MQC (900)			
Day 1	944.93	3.93	4.99
Day 2	893.89	3.96	− 0.68
Day 3	831.45	5.06	− 7.62
HQC (1800)			
Day 1	1741.85	6.71	− 3.23
Day 2	1699.37	10.91	− 5.59
Day 3	1820.43	8.26	1.13
Interassay ($n = 18$)			
LQC	428.46	2.21	− 4.79
MQC	881.70	5.15	− 2.03
HQC	1745.49	3.75	− 3.03

RSD, relative standard deviation.

Table III

Stability Data of T and ODT in Human Plasma ($n = 4$)

Storage condition	Level (ng/mL)	Tramadol			ODT		
		(ng/mL)	% RSD	% Bias	(ng/mL)	% RSD	% Bias
Room temperature	450	442.93	3.43	−1.57	442.26	1.18	−1.72
	900	839.2	4.78	3.61	837.30	7.40	2.30
Refrigerator condition (4°C)	450	393.27	2.58	−12.61	418.41	1.18	−7.02
	900	915.4	6.21	13.02	866.0	8.00	5.80

Table IV

Comparison of Selected Analytical Methods Developed for Determination of T and ODT by HPLC−DAD

Analytes	Samples	Extraction procedure	mobile phase	Run time (min)	LOD (ng/mL)	LOQ (ng/mL)	Recovery (%)	Ref.
T	Rabbit plasma	LLE; ethylacetate	Acetonitrile:phosphate buffer (25:75), pH 3.0	10	250	400	90.40	[33]
T	Human plasma	LLE; ethylacetate	Acetonitrile:phosphate buffer (25:75), pH 3.0	10	200	350	97.7	[34]
T	Saliva	LLE; hexane:ethylacetate (4:1, v/v)	Acetonitrile:phosphate buffer buffer (40:60), pH 3.0	10	100	250	94.70	[35]
T and ODT	Human plasma	LLE; MTBE	Acetonitrile:phosphate buffer buffer (50:50), pH 5.5	10	100	250	83.51 for T and 78.72 for ODT	Present method
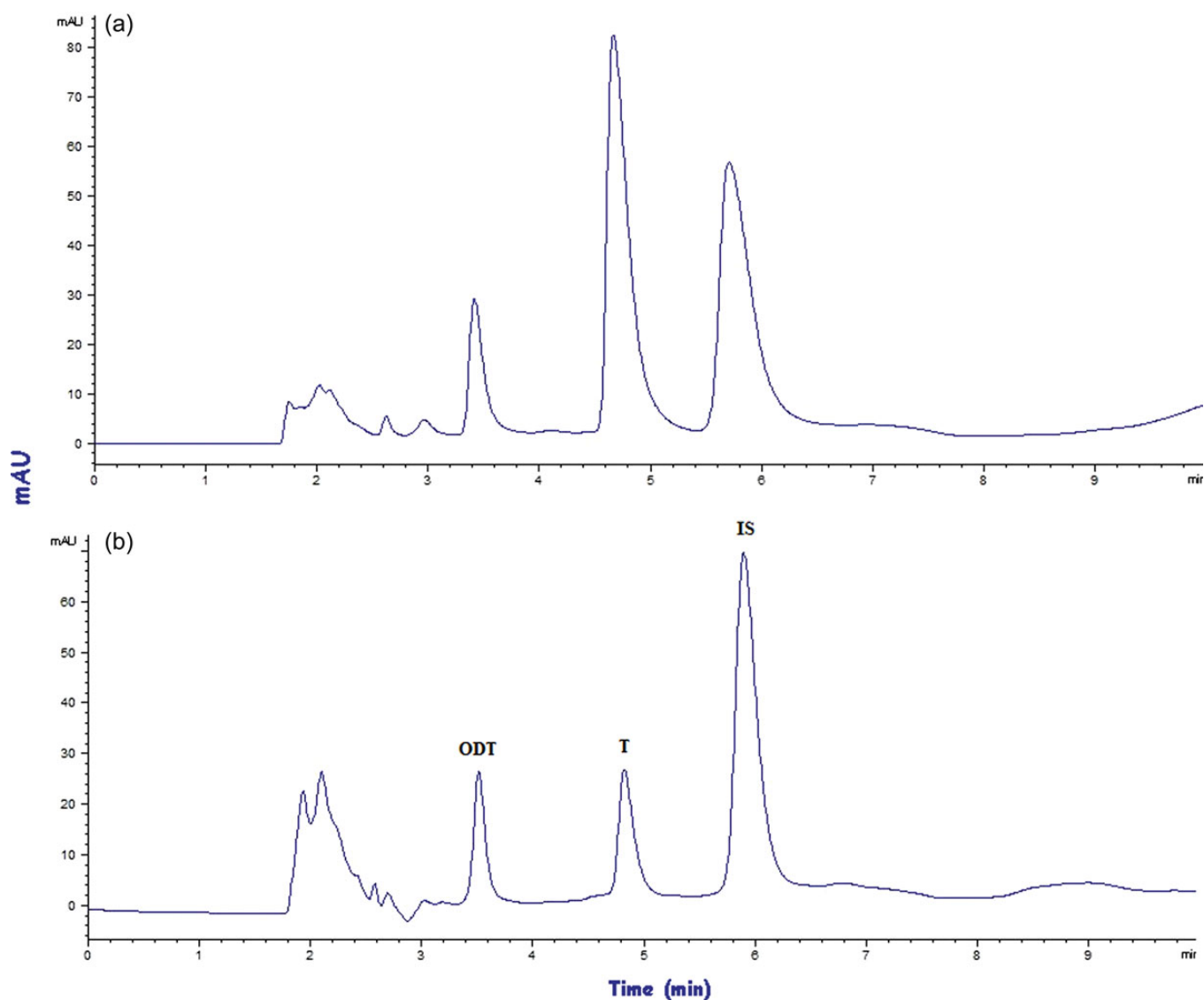
Figure 3. HPLC chromatograms after analysis of (a) unknown sample provided by CAP and (b) LQC (450 ng/mL).

method may be a useful analytical procedure for the fields of clinical and forensic toxicology applications.

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