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mono-Brominated-Propyphenazone as a New Derivatization Reagent for High Performance Liquid Chromatography of Ephedrine with UV-Detection

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Abstract. *mono*-brominated-propyphenazone was used as a derivatization reagent for the detection and quantification of ephedrine by high performance liquid chromatography using Zorbax-XDB C18 column, and 0.02 M sodium acetate (pH 4.4): acetonitrile (52:48 v/v) as mobile phase and UV detection at 280nm. The cited reagent reacts with the secondary amino group of ephedrine in acetone using anhydrous potassium carbonate as hydrobromide acceptor. The reaction was completed within 20 minutes in 100 µL acetone with heating at 90 ± 5°C in a capped mini reaction vial. The stoichiometry of the reaction was one-to-one mol from each *mono*-brominated-propyphenazone and ephedrine. The linear concentration range of ephedrine was 30 to 900 ng per injection (50 µL). The detection limit was 9 ng/injection (50 µL). The derivatized ephedrine was synthesized and confirmed with spectral analysis. This method was applied for determination of spiked ephedrine in human urine and after extraction with charcoal column using methanol: 0.2 M NaOH (20:0.2, mL:mL) as eluant.

Keywords: Candesartan; Hydrochlorothiazide, Tablets, High performance liquid chromatography

Introduction

Ephedrine (Eph) is a sympathomimetic drug which is indicated primarily to counteract the hypotensive effects of spinal or other types of nontopical conduction anesthesia, and commonly co-formulated in anti-

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cough syrups[1]. Eph has very low UV-absorption[1]. It has been reported that Eph is readily absorbed after oral or percutaneous administration and metabolised by N-demethylation to norephedrine (phenylpropanolamine) and by oxidative deamination followed by conjugation. About 90% of a Eph dose is excreted in the urine in 24 h, with about 55 to 75% of the dose as an unchanged drug, 8 to 20% as norephedrine, and 4 to 13% as deaminated metabolites[1]. Two reports have been adopted to enhance its UV-absorption. These include the use of 4-(4,5-diphenyl-1H-imidazole-2-yl) benzoyl chloride[2] and 3,5-dinitrobenzoyl chloride[3] as derivatization reagents for high performance liquid chromatography (HPLC)-UV determination of Eph. In addition, different fluorogenic derivatizing reagents have been reported for determination of Eph, including; 9-fluorenylmethyl chloroformate[4], 5-dimethyl-aminonaphthalene-1-sulfonyl chloride[5], and ophthaldialdehyde[6]. On the other hand, *mono*-brominated-propyphenazone (BMP), has been synthesized by Meister Lucius and Brüning (1907)[7], and used as a coupling agent for the synthesis of famprofazone and famprofazone analogues[8,9]. Besides, BMP has also been used as a derivatization reagent for high performance liquid chromatography of captopril with UV detection at 254 nm and 284 nm[10]. The cited reagent (BMP) has been found to react with mercapto and amino groups[10]. In this work, BMP as a derivatization reagent reacted with the secondary amino group of Eph (Fig. 1). The derivatized Eph has shown maximum UV-absorption at 267 nm and 280 nm (ε = 7090 g $cm^{-1}L^{-1}$), which enable its determination in urine. The reaction conditions were optimized to ensure complete derivatization of Eph.

Experimental

Materials

All solvents used were of HPLC grade, Merck, Darmstadt, Germany. Activated Charcoal, NORIT SA 2, decolorizing, and Silicagel for column chromatography (0.2-0.5 mm pore diameter ca 4 nm) was purchased from from Acros Organics (Geel, Belgium). Propyphenazone was obtained from Caeser & Loretz (Hilden, Germany). TLC silica gel 60 F254 aluminum sheets were purchased from E-Merck (Darmstadt, Germany). All other solvents used were analytical grade. *mono*- BMP was synthesized from propyphenazone and bromine, according to Meister, Lucius, Brüning in Höchst, 1907[7], recrystallized from

chloroform: diethylether (1:2 v/v), and tested for its purity by TLC and HPLC analysis. ^{1}H NMR and IR analysis confirmed the structure of synthesized BMP.

Apparatus

Alliance Water separations module 2695 and Waters 2996 Photodiode array detector were set to 280nm, and column heater was set to 30 ± 2°C. Chromatograms were integrated with Empower software. HPLC column was Agilant Zorbax-XDB C18 column (4.6 mm i.d. x 25 cm, 5 miron particle diameter). UV-Visible spectrophotometer; UV-1700 PharmaSpec, with UV probe, version 2.00 software, Shimadzu, Japan. FT-IR Spectrometer system, Spectrum GX, with Spectrum GX1 V5.0 software, from Perkin Elmer. GC/MS Clarus 500 Gas Chromatograph, Clarus 500 Mass spectrometer (Perkin Elmer, USA). Software controller/integrator; TurboMass, version 4.5.0.007, Perkin Elmer, USA). Elite 5MS GC capillary column, 30 mm x 0.25 mm x 0.5 um (Perkin Elmer, USA). Carrier gas; Helium, flow; 2 mL/min., 32 p.s.i., flow initial 55.8 cm/sec., split; 1:20. Inlet line temperature; 250°C, source temperature; 170°C, trap emission; 100°C, electron energy; 70 eV. Column temperature program; 140°C for 5 min, to 280°C (rate, 20°C / min), hold for 10 min. Injector; 250 C. MS scan from 50 to 650 m/z. Screw-capped borosilicon mini-reaction vials 1ml, v-shaped, with tetrafluoroethylene (TFE) liners were used for derivatization reaction (Alltech, GmbH, Unterhaching, Germany). ^{1}H-NMR was recorded on JEOL JNM-EX 270 MHz spectrometer (Tokyo, Japan) sample, and was dissolved in $CDCl_3$ using TMS as internal standard.

Mobile Phase

Mobile phase was prepared by mixing 520 mL 0.02 M sodium acetate (adjusted to pH 4.4 with 100% acetic acid) with 480 mL acetonitrile, filtered, degassed, and pumped at a flow rate of 1.0 mL/min.

Solutions

BMP reagent (3.23 mM) was prepared by dissolving 10 mg BMP in 10 mL acetone in volumetric flask (protected from light).

Eph anhydrous RS solution (3.23 mM) was prepared by dissolving 53.4 mg Eph anhydrous in 100 mL acetone.

Synthesis of Mono-Brominated-Propyphenazone (BMP)

[*5-(Bromomethyl)- 4-isopropy-1-methyl -2-phenyl-1H-pyrazol-3(2H)-one*], $C_{14}H_{17}$ Br N_2 O, was synthesized from propyphenazone and bromine, according to **Meister, Lucius, Brüning in Höchst**, 1907[7], with some modifications to increase yield and purity as follows; about 10g propyphenazone was dissolved in 25 mL CH_2Cl_2 in 100 mL round flask fitted with dropping funnel; it was kept over an ice bath and bromine solution (6.95 g dissolved in 5 mL CH_2Cl_2) was added drop-wise and very slowly with the aid of stirring for 1 hr. The brown color of the bromine faded rapidly upon its addition. An aliquot of 30 mL 10% cold aqueous sodium carbonate solution was added with vigorous shaking. The organic phase was separated and dried with Na_2SO_4, filtered, and concentrated under vacuum at 40°C until the volume was about 10 mL. The reaction mixture was cooled to room temperature and about 15 mL diethyl ether was added. This solution was left to stand in stoppered flask at room temperature in the dark. The colorless crystals which formed were separated and washed with cold diethyl ether (about 5°C). The modifications made to the reported method included, (a) the use of cold sodium bicarbonate aqueous solution to avoid formation of any 3-hydroxymethyl-propyphenazone; (b) using diethyl ether instead of dichloromethane to get more yield of BMP crystals; and (c) using cold diethyl ether for crystallization instead of dichloromethane at room temperature to get more pure crystals of BMP.

Synthesis of Reaction Product with BMP (MP-Eph)

[*4-isopropyl-2-methyl-3-[N-methyl-N-(α-methyl-β-hydroxy-phenethyl) aminomethyl]-1-phenyl-3-pyrazolin-5-one*], C_{24} H_{31} N_3 O_2, Into a 50 mL round flask, 1 g Eph anhydrous (0.006 mol) was dissolved in 5 mL acetone containing 2 g anhydrous potassium carbonate. This solution was heated to about 60°C, and 1.8 g of *mono*-brominated-propyphenazone (dissolved in 5 mL acetone, 0.006 mol) was added drop-wise. A double-sided water condenser was constructed and the reaction mixture was refluxed with stirring for 20 min. Finally, the reaction mixture was cooled and dried under vacuum at 50°C. The residue was washed with about 20 mL acetone, and about 100 mL water, subsequently. The residue was dried in vacuum desiccator over P_2O_5 (yield, 99.4%).

Derivatization of Eph

Into a borosilicate screw-capped mini-reaction vial of 1.8 mL capacity, a volume spanning the range of 4-100 µL Eph reference standard solution, and 100 µL 3.23 mM BMP solution were transferred and dried over nitrogen stream, then mixed with about 2 mg anhydrous K_2CO_3. An amount of 100 µL acetone was added, and the vial was capped and half-inserted in block heater adjusted at 90 ± 5°C for 20 min. The reaction mixture was cooled, evaporated to dryness over nitrogen stream, reconstituted in 1500 µL acetone and shaken for 1 min. Twenty-five µL were finally injected for HPLC analysis (Rt = 12.18 min). The procedure was repeated five times for seven different concentrations from EPH reference standard solution.

Urine Extraction

Extraction column was prepared by mixing 1.5 g activated charcoal with 2 g silica gel for column chromatography, and packed in a 50 mL glass syringe. One liter of urine was collected from a healthy person, free from drugs and food preservatives, and was spiked with 1.066 mg anhydrous EPH. An aliquot of 2.5 mL from this solution was extracted with the extraction column using methanol: 0.2M NaOH (20: 0.2, mL: mL) as eluant, into two portions. The eluant was evaporated to dryness under vacuum and the residue was quantitatively transferred to the reaction vial. The solution was then gently dried under a stream of nitrogen gas, reconstituted in 60 µL acetone and 100 µL 3.23 mM BMP solution. This mixture was mixed with ca. 2 mg anhydrous K_2CO_3, and the vial was capped, half-inserted in a block heater adjusted at 90 ± 5°C for 20 min, cooled, evaporated to dryness over nitrogen stream, reconstituted with 300 µL acetone, and a volume of 50 µL was injected for HPLC analysis. This procedure was repeated three times using three different concentrations of spiked urine (0.5 mg, 1.0 mg, and 1.5 mg EP/L urine) parallel with non-spiked urine samples from the same person.

Results

Synthesis of BMP and MP-EPH

The crystallized BMP from diethyl ether was pure and did not need further purification. The structure and purity was in accordance with the

data reported by Meister, Lucius, Brüning in Höchst (1907)[7]. The obtained crystals were dried overnight in vacuum desiccator over P_2O_5, in the dark (yield, 89%) (*TLC*; toluene: acetone: ethanol, 9: 0.5: 0.5 v/v, R_f 0.73, *melting point*; 98°C, *MS data*; m/z = 308(45%), 310(47%), 293(58%), 295(62%), 229(50%), 77(100%).

The purity of MP-EPH reaction product was verified and confirmed by thin-layer chromatography (TLC), infrared spectrometry (IR) and high-performance liquid chromatography (HPLC). (*TLC*; toluene: acetone: ethanol, 9: 0.5: 0.5 v/v, R_f 0.40, *MP*; 225°C,). The GC-MS data was generated using the chromatographic and mass spectrometer settings mentioned before. The retention time was 22.9 minutes, and the characteristic mass fragments with major abundance were labeled as shown in Fig. 1. *IR* data are shown in Fig. 2. The method performance parameters were used to confirm completeness of the reaction (Table 1). The ^{1}H-NMR data are summarized in Table 2.

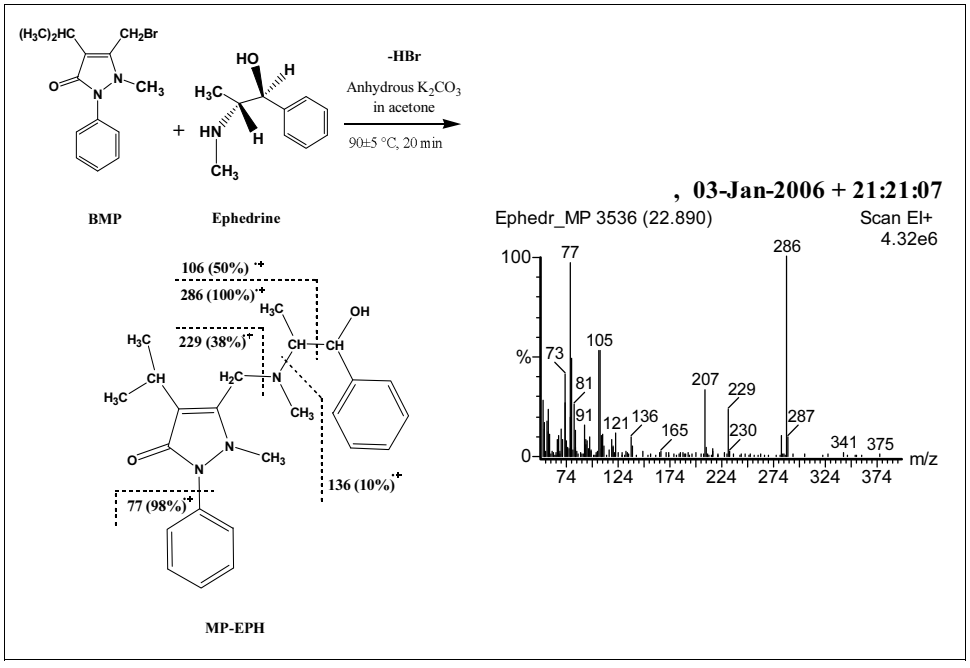


Fig. 1. Interpreted mass spectrum of MP-EPH, m/z (abundance %).

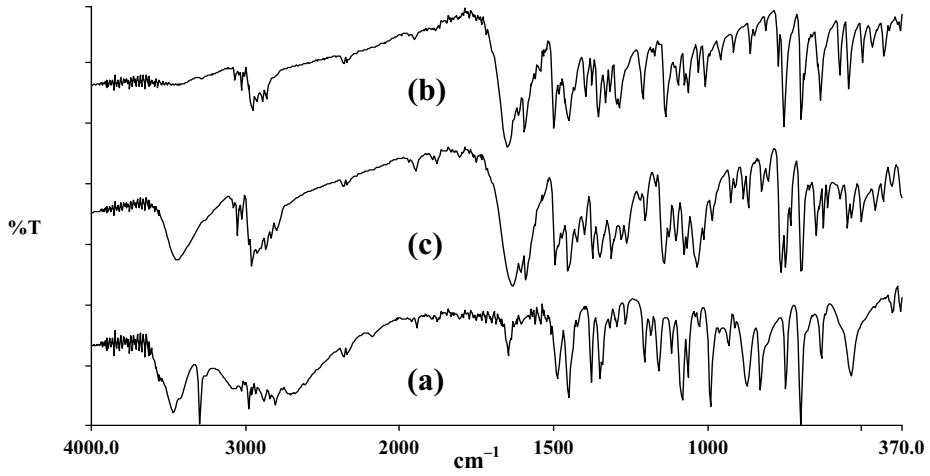


Fig. 2. Infrared spectra of Eph anhydrous (a), BMP (b), and MP-Eph (c), prepared in KBr discs.

Table 1. System suitability data of BMP and MP-Eph[†].

Substance	Rt (min) ± SD	K' ± SD	N ± SD	As ± SD	R ± SD
BMP	9.00 ± 0.0	3.5 ± 0.004	16200 ± 0.2	1.0 ± 0.1	5 ± 0.1
MP-Eph	12.24 ± 0.0	5.12 ± 0.007	15200 ± 0.2	1.1 ± 0.1	

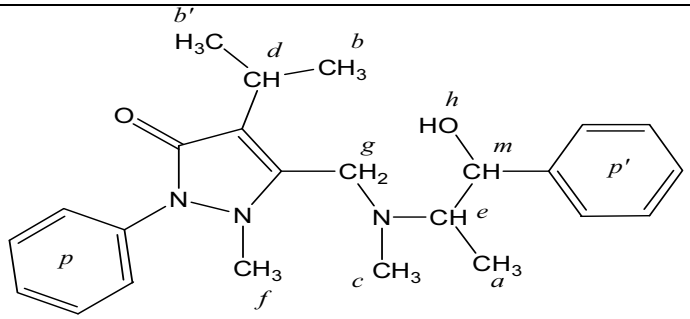
[†] K' capacity factor; R, resolution; N, USP-plate count, and As, peak asymmetry.

Factors Affecting Derivatization

The completeness of the reaction was monitored referring to the HPLC and TLC analysis.

The reaction stoichiometry was 1:1, BMP to Eph. The excess reagent peak monitored by HPLC was eluted at 9.0 minutes and the reaction product was eluted at about 12.0 minutes. Upon derivatization of Eph with BMP using 1:1 molar ratio, there was no excess reagent or Eph peaks, and only one peak eluted at 9.0 minutes corresponded to the derivatization product was observed (Fig. 3). Fully derivatized Eph was achieved upon heating at 90 ± 5°C for 20 min. At an elevated temperature (> 1 10°C) an unknown peak close to MP-Eph peak was observed (Fig. 3). The reaction time course was optimized as illustrated in Fig. 4.

Table 2. ^{1}H-NMR data of MP-Eph (270 MHz) reference to TMS in $CDCl_3$.



Proton	σ (ppm)	Integration (nH)
a	1.15 (d)	3H
b&b'	1.30 (d)	6H
c	2.27 (s)	3H
d	2.75 (m)	1H
e	2.91 (m)	1H
f	2.93 (s)	3H
g	3.57 (s)	1H
h	2.00 (s)	1H
m	4.78 (d)	1H
p&p'	7.08 – 7.65 (m)	10H

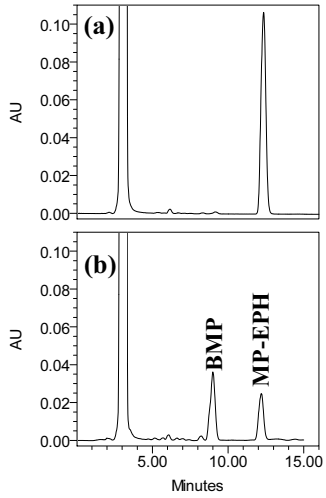


Fig. 3. Representative chromatogram of derivatized standard Eph solution, 888 ng/injection (50 µL), with BMP in a molar ratio of 1: 1 (a), and in a molar ratio of 0.25: 1, respectively (b).

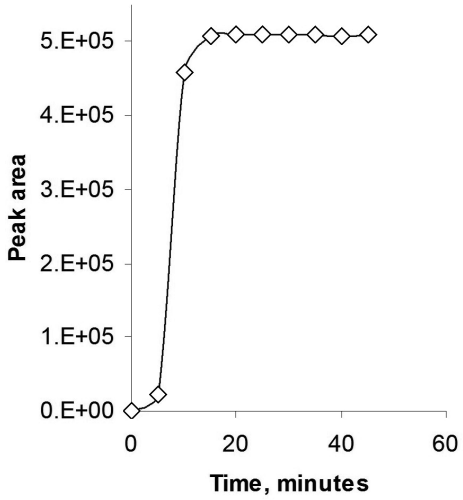


Fig. 4. Reaction time course of ephedrine (0.2 μg/injection (50 μL)) with BMP at 90 ± 5°C.

Method Validation

The calibration curve for Eph was investigated over the range of 30-900ng per injection (50μL) ($r^2 = 0.999$). The regression equation was;

injected concentration (in nanogram) = (3.89)(10^{-4}) Peak area + 1.58.

The limit of detection was 9 ng/injection (50 μL) at a signal-to-noise ratio of 3:1, and the limit of quantification was 30ng/injection (50μL) at a signal-to-noise ration of 9:1. The RSD of peak area of repetitive injection (three days) of derivatized EPH (0.5μg/injection (50μL)) was 0.4. The performance parameters were listed in Table 1.

Discussion

Eph has insignificance UV absorption at its maximum wave length[1]. To enable its detection and monitoring in urine, it was necessary to enhance its UV absorption by adopting a stoichiometric derivatization using BMP.

Synthesis of BMP

To obtain high yield and better purity of BMP, anhydrous solvents were used to avoid formation of 3-hydroxymethyl-propyphenazone,

which is the hydrolysis product of BMP. A cold solution of sodium carbonate was used to remove the excess bromine. The crystallized BMP from diethyl ether was pure and did not need further purification. The dried BMP powder should be protected from light and moisture.

Factors Affecting Derivatization

The type and amount of solvent used as reaction media were studied. The derivatization reaction was more preferable to proceed in a minimal amount of dry acetone than chloroform, ether, or dichloromethane. However, acetone or acetonitrile have shown the same result. Anhydrous potassium carbonate powder was used as hydrobromide acceptor; however, trimethylamine, dimethylamine, or pyridine was not suitable as several unknown reaction products were observed. The reaction stoichiometry was 1:1, BMP to Eph. The excess reagent peak was monitored by HPLC at about 9.0 min, and the reaction product was eluted at about 12.0 min. Upon derivatization of Eph with BMP using 1:1 molar ratio, there was no excess reagent or Eph peaks, and only one peak eluted at 9.0 min corresponding to the derivatization product was observed (Fig. 3). To improve the resolution between the reaction product and BMP, the derivatized Eph reaction mixture was dried under a stream of nitrogen gas and reconstituted with 100μL methanol, heated for 5 min at about 55°C, cooled and injected for HPLC analysis. BMP was completely abolished upon using methanol as a solvent in the reaction media. Because of this, methanol was not used as a solvent.

Fully derivatized Eph was achieved upon heating at 90 ± 5°C for 20 min. At an elevated temperature (> 110°C) an unknown peak close to MP-Eph peak was observed (Fig. 3). The reaction time course was optimized as illustrated in Fig. 4.

The amounts of BMP used were sufficient for the claimed amount of standard EPH solution, and much more amounts of BMP have no effect on the amount of product expected.

Chromatographic Method Optimization

The cited mobile system was found to be suitable for the analysis of derivatized EPH, using Agilant Zorbax-XDB C18 column maintained at 30 ± 2°C. However, band broadening or bad resolution was observed

upon using Zorbax-TMS or Zorbax-CN columns. Peak purity was confirmed by matching the diode-3D chromatogram of synthesized MP-Eph with that of derivatized standard Eph solution.

The system suitability was tested by 6 repetitive injections of derivatized EPH standard solution (266 ng/injection (50 µL)). The system suitability data showed an acceptable RSD of resolution, retention time, theoretical plate count, and capacity factor (Table 1).

Method Validation

The calibration curve for Eph was linear over the range from 30 to 900ng per injection (r^2 = 0.999). The RSD of peak area of repetitive injection (three days) of derivatized Eph (0.5 µg/injection (50 µL)) was 0.4. The developed method was selective since it was able to discriminate between derivatized EPH and BMP by enough resolution (R=5) as shown in Table 1.

Extraction of Ephedrine from Urine

The recovered amount of Eph from spiked human urine was found to be 100 ± 0.5%. The amount of BMP required for complete derivatization of urine extract was increased two times compared with the amount used for derivatization of standard Eph solution. The amount of final dilution was 300 µL. The intra-day precision of the recovered Eph from urine was not more than 2.6% (RSD value).

Figure 5 represents the chromatogram of derivatized spiked-urine extract. The peak monitored at retention time of 12.0 min was not detected in blank urine extract treated with the same procedure.

Structural Confirmation of MP-EPH

The derivatized EPH prepared was investigated by UV scan, ^{1}H-NMR, IR, GC-MS, TLC and HPLC.

Table 2 shows the ^{1}H-NMR data of MP-EPH, which confirm alkylation at NH- group. The IR data showed C=O band at 1634 cm^{-1} and 1648 cm^{-1} for MP-EPH and BMP, respectively, -O-H band at 3449 cm^{-1} and 3470 cm^{-1} for MP-EPH and EPH, respectively (Fig. 2). Both MP-EPH and BMP peaks were scanned by diode array and showed λ_{max} of 288 and 294 nm, respectively. The calculated molar absorptivities

were 7090 and 7465g $cm^{-1}L^{-1}$ for MP-EPH and EPH, respectively. This confirms the production of derivatized EPH with an acceptable absorptivity. The GC-MS data showed characteristic fragments that derived from propyphenazone (m/z, 229, 38%), and base peak of m/z, 286 that confirm the EPH moiety (Fig. 1).

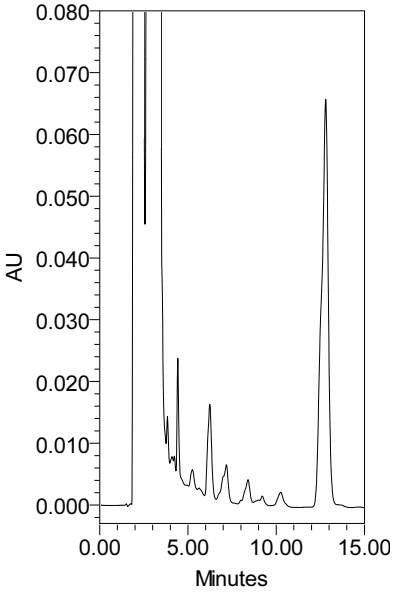


Fig. 5. Representative chromatogram of derivatized spiked - urine extract.

Conclusion

Conclusively, *mono*-brominated-propyphenazone (BMP) could be used as a derivatization reagent for HPLC determination of Eph in urine with UV detection. BMP is reactive towards basic secondary amino functional group. The sensitivity of this method is much less than that using fluorescence detection, but it is of value in case UV detection is used with other co-eluted drugs.

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٣-بروموميثيل-بروبيفينازون كعامل اشتقاق جديد لتحليل الإفدرين بكروماتوجرافيا السوائل ذات الأداء العالي مع الكشف بالأشعة فوق البنفسجية

علاء الدين محمود خضر و فايدة حسن بامانع

قسم الكيمياء الصيدلانية، كلية الصيدلة، و قسم الكيمياء الطبية، كلية الطب

جامعة الملك عبدالعزيز

جدة – المملكة العربية السعودية

المستخلص. لقد تم استخدام ٣-بروموميثيل-بروبيفينازون (ب م ب) بروبيفينازون كعامل اشتقاق جديد لتحليل الإفدرين بكروماتوجرافيا السوائل ذات الأداء العالي، مع الكشف بالأشعة فوق البنفسجية مستخدماً عمود فصل زورباكس-إكس دي بي-ك١٨، ووسط متحرك يتكون من ٠،٠٢ مول خلات الصوديوم (اس هيدروجيني ٤،٤) و مذيب أسيتونيتريل (بنسبة ٥٢: ٤٨ حجم/حجم)، مع الكشف بالأشعة فوق البنفسجية عند طول موجي ٢٨٠ نانوميتر. لقد ثبت تفاعل عامل الاشتقاق المذكور مع مجموعة الأمين الثنائية بالإفدرين في مذيب الأسيتون، مع استخدام كربونات الصوديوم اللآمائي لاستقطاب حمض الهيدروبروميد الناتج من التفاعل. يكتمل هذا التفاعل في خلال ٢٠ دقيقة في ١٠٠ ميكروليتر أسيتون مع التسخين عند درجة حرارة ٩٠±٥ مئوية في قوارير زجاجية مخصصة لذلك. ولقد ثبت أن النسب المتفاعلة هي واحد مول إلى واحد مول من كل الـــ ب م ب و الإفدرين. ووجد

أن العلاقة الكمية الخطية للأفدرين تقع في نطاق ٨٨ إلى ٨٨٨ نانوجرام/الحقنة الواحدة، وأقل كمية يمكن كشفها تساوي ٩ نانوجرام/الحقنة. لقد تم تشييد الإفدرين المشتق، وتأكيد التركيب الكيميائي للناتج بطرق تحليلية طيفية. ولقد تم تطبيق الطريقة في تحليل الإفدرين المفروز في البول البشري، بعد عملية الاستخلاص باستخدام الغول الميثيلي، مع هيدروكسيد الصوديوم (بنسبة ٢٠: ٠,٢ ملي/مللي) على عمود الكربون المطحون.؛