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Article

Determination of Bupropion Using Liquid Chromatography with Fluorescence Detection in Pharmaceutical Preparations, Human Plasma and Human Urine

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A novel pre-column derivatization reversed-phase high-performance liquid chromatography with fluorescence detection is described for the determination of bupropion in pharmaceutical preparation, human plasma and human urine using mexiletine as internal standard. The proposed method is based on the reaction of 4-chloro-7-nitrobenzofurazan (NBD-Cl) with bupropion to produce a fluorescent derivative. The derivative formed is monitored on a C18 (150 mm × 4.6 mm i.d., 5 μm) column using a mobile phase consisting of methanol–water 75:25 (*v*/*v*), at a flow-rate of 1.2 mL/min and detected fluorimetrically at $\lambda_{ex} = 458$ and $\lambda_{em} = 533$ nm. The assay was linear over the concentration ranges of 5–500 and 10–500 ng/mL for plasma and urine, respectively. The limits of detection and quantification were calculated to be 0.24 and 0.72 ng/mL for plasma and urine, respectively (inter-day results). The recoveries obtained for plasma and urine were 97.12% ± 0.45 and 96.00% ± 0.45, respectively. The method presents good performance in terms of precision, accuracy, specificity, linearity, detection and quantification limits and robustness. The proposed method is applied to determine bupropion in commercially available tablets. The results were compared with an ultraviolet spectrophotometry method using *t*- and *F*-tests.

Introduction

Bupropion (BUP) hydrochloride is chemically known as (±) -2-*tert*-butyl-amino)-3′-chloropriophenone hydrochloride (1). It is an aminoketone used as second-generation antidepressant and non-nicotine aid to smoking cessation (2). BUP is presumed to be a dopamine-norepinephrine reuptake inhibitor (3). The chemical structure of BUP is presented in Figure 1.

Various analytical techniques have been employed for the determination of BUP in pharmaceutical preparations and biological fluids, such as derivative spectrophotometry (4), thin-layer chromatography (5), high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (6–12), gas chromatography (13, 14), liquid chromatography–tandem mass spectrometry (15, 16) and electrokinetic chromatography (17).

To the best of our knowledge, no pre-column derivatization method has been reported thus far for the determination of BUP. It is obviously known that fluorescence detection is more sensitive than the other spectrophotometric studies.

Because of this, this study attempted to develop a method for the determination of BUP. It was first derivatized by 4-chloro-7-nitrobenzofurazan (NBD-Cl) and optimum conditions were achieved for the quantification. The method was then fully validated according to the International Conference of Harmonization (ICH) guidelines (18). It was essential to establish an assay with a limit of detection (LOD) in the low ng/mL range. Short separation times and high sensitivity, without compromising the specificity, are the primary advantages of such a technique.

Experimental

Materials and reagents

BUP and mexiletine as internal standard (IS) (all > 98% purity) were supplied from Sigma (St. Louis, MO). Pharmaceutical preparation, Wellbutrin tablets (150 mg) was provided from Glaxo Smith Kline (Istanbul, TR) in a local pharmacy. NBD-Cl and other chemicals were purchased from Merck (Darmstadt, Germany). All chemicals and solvents were of analytical grade.

Venous blood samples were collected from healthy volunteers into a polyethylene tube containing ethylenediaminetetraacetic acid, which it was centrifuged at 4,500 rpm for 35 min. Human plasma was separated from whole blood by a pipet. Urine samples were transferred into 50-mL polyethylene tubes. Plasma and urine samples were kept in a refrigerator at −20°C until analysis.

Instrumentation

The fluorescence spectrum of BUP was recorded by a RF-1501 Model spectrofluorimeter. The UV-visible spectrophotometer UV-160A with 1-cm matched quartz cells, the HPLC system consisting of a Model LC 20 AT solvent delivery system with an SIL-20AHT autosampler with a 5-mL loop and the RF-10AXL fluorescence detector were all provided from Shimadzu (Kyoto, Japan). An analytical Inertsil C18 column (150 mm × 4.6 mm i.d., 5 mm) with a guard column (4 mm × 3 mm i.d., 5 mm) packed with the same material was purchased from ATAS GL (Eindhoven, Netherlands). Ultra-pure water was prepared by using an of aquaMAX Model from Young Instruments (Anyang, South Korea).

Procedures

Mobil phase and HPLC conditions

A mobile phase consisting of methanol–water [75:25 (v/v)] was utilized. A flow-rate of 1.2 mL/min and injecting volume of 5 mL were utilized during the analysis. The fluorescent

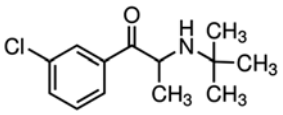

Figure 1. Chemical structure of BUP.

detector was set at 458 and 533 nm for the excitation and emission wavelength, respectively.

Solutions

HPLC method

Stock standard solutions of BUP and IS were prepared in methanol at a concentration of 100 μg/mL and stored at +4°C. These were diluted by using methanol to give appropriate working (10 μg/mL) solution. Standard solutions were prepared in a 0.5-mL volume of human plasma and urine to yield final concentrations of 5–500 and 10–500 ng/mL, respectively. NBD-CI was freshly prepared as 1.5×10^{-2}M in methanol. Borate buffer 0.025M (sodium tetraborate) was prepared by adjusting the pH to 9.0 with 0.1N sodium hydroxide and 0.1N hydrochloric acid solutions.

Method validation

The proposed method was validated according to the ICH Guidance Documents (18). The system suitability, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), recovery, robustness and specificity parameters were analyzed for the validation testing.

Accuracy and precision

The accuracy and precision of BUP-NBD derivatives were tested at three concentration levels over three consecutive days and expressed by relative mean error (RME %) and relative standard deviation (RSD %), respectively.

Linearity

Calibration curves of BUP-NBD derivatives were constructed by linear regression using the internal standard technique. The plot of peak area ratios ($rPN = PN_{BUP}/PN_{IS}$) versus concentrations of the mentioned compound were employed to find out the linearity parameters.

Limits of detection and quantification

LOD and LOQ were calculated according to the following equations: $LOD = 3.3\sigma/a$ and $LOQ = 10\sigma/a$; where σ is the standard deviation of the intercept of regression equation and a is the slope of the calibration curve.

Recovery

Recovery from plasma, urine and tablets was carried out by the standard addition method. Different concentrations of pure drug were added to a known pre-analyzed formulation sample and the concentrations of BUP-NBD in the relevant matrix were determined by using the proposed method.

Robustness

Robustness of the method was performed to evaluate the influence of small but deliberate variations in the chromatographic conditions for the determination of BUP derivation with NBD-Cl. Flow-rate and mobile phase composition were examined for this purpose.

Specificity

Studies for probable interfering substances of BUP were examined by testing some matrix agents often used in tablet formulations to examine the specificity.

Stability

The stability of BUP was tested in various media and conditions. These were examined by injecting into the HPLC and evaluated by comparing the results to the rPN values of BUP and IS.

UV spectrophotometry (comparison method)

One hundred milligrams BUP were accurately weighed and dissolved in 100 mL of methanol. A 10-mL volume of the solution was adjusted to 100 mL with methanol to prepare the stock solution. Standard solutions were obtained by diluting the stock solution for the preparation of calibration curves in the concentration range of 2–20 μg/mL. The absorbance of BUP was measured at 248.5 nm, which is the maximum value at which BUP absorbs monochromatic light.

Tablets

Twenty tablets were weighed and finely powdered and portions equivalent to 100 mg BUP were transferred into a 100-mL volumetric flask; 50 mL methanol was added and shaken thoroughly to dissolve. The solution was brought to volume, mixed well and centrifuged, and the supernatant was used to prepare solutions of 1 ng/mL of BUP using methanol as the diluent. This solution (10 μg/mL) obtained by dilution of supernatant-was used to set up the concentrations in the range of calibration studies.

Extraction from plasma and urine

All frozen plasma and urine samples (1 mL) were thawed just before the experiment and BUP was added in the concentrations ranges 5–500 and 10–500 ng/mL, respectively. A fixed amount (100 ng) of IS was enhanced and vigorously shaken to construct the calibration equations. Then, 1 mL of 1M NaOH was added, and shaken by a vortex for 60 s and extracted with 3 mL of ethyl acetate, then centrifuged at 4,500 rpm for 35 min. The organic layer (3.5 mL) was transferred to a 5-mL glass tube and evaporated to dryness under a nitrogen stream at 40°C. One hundred microliters buffer and 100 μL NBD-Cl solutions were added to the residue and the mixture was kept

at 70°C for 20 min in a water bath. The mixture was cooled and acidified with 100 μL of 1N HCl. The associated compound was extracted from plasma and urine twice with 2.5 mL chloroform and the organic layer was transferred to a tube. The organic phase was dried on anhydrous sodium sulfate. A 4.5-mL aliquot of the extract was evaporated under nitrogen at 45°C. The residue was then dissolved in 1 mL of the mobile phase and filtered through a 0.2-μm membrane filter. Typically, 5 μL aliquots of this solution are used for HPLC determination.

Incured BUP reanalysis

The incured sample reanalysis was applied to the method considering the Food and Drug Administration (FDA) suggestions (19). Original BUP solutions were added 1 mL into plasma and urine at the concentrations of 5, 25, 50, 100, 250 and 500 ng/mL for plasma and 10, 25, 50, 100, 250 and 500 ng/mL for urine, respectively. They were vigorously shaken and BUP contents were extracted by employing a liquid–liquid extraction method utilizing ethylacetate. Incured BUP reanalysis was performed to determine the reproducibility of the method. The percent difference was calculated as follows:

%difference = reassay value − original value / average of reassay and original value

Results and Discussion

Derivatization

NBD-Cl, an activated halide derivative, was first introduced as a fluorogenic reagent for the determination of secondary and primary amines (20). Thus, it is possible to detect low amounts by the use of pharmaceutical-NBD-Cl adduct. Some studies have been performed subjecting certain pharmaceuticals to derivatization of with NBD-Cl employing spectrofluorimetry (21, 22) and HPLC (23–27).

BUP contains a secondary aliphatic amino group that was found to react with NBD-Cl in an alkaline buffer medium, yielding a highly yellow fluorescent product that exhibited its highest fluorescence intensity at 458 nm after excitation at 533 nm.

To investigate the optimum conditions, certain types of buffer and the effect of the pH on the formation of BUP-NBD was examined by varying the pH from 7 to 10. Borate buffer gave a high yield (as indicated by the fluorescence intensity of the derivative) and the best yields were obtained at pH 9. The effect of time and temperature on the reaction was also studied by varying the temperature from 50 to 80°C. The best result was obtained at 70°C within 20 min (Figure 2).

The hydrolysis product of NBD-Cl (NBD-OH, 4-hydroxy-7-nitrobenzofurazan) may cause problems by interfering with the main product. Therefore, the reaction mixture is absolutely acidified (pH 2, by adding 100 μL of 1N HCl) before the measurement of the fluorescence intensity, as stated earlier (28).

Chromatographic conditions

Several parameters were examined for the optimization of HPLC analysis of the BUP-NBD associated compound. The first attempt was to find out the consistency of the mobile phase. The isocratic mode was preferred and different mobile phases were employed to develop a rugged and suitable HPLC method for

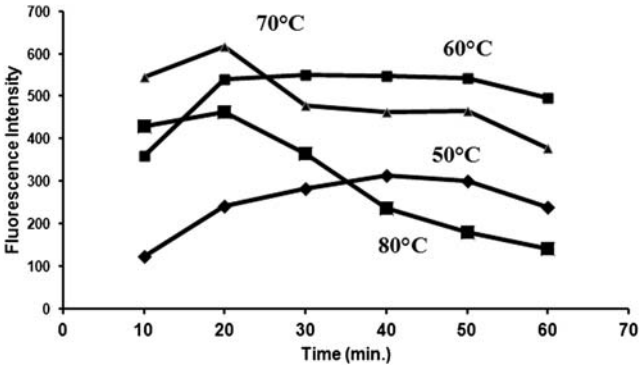

Figure 2. Effects of time and temperature on the reaction of BUP with NBD-Cl.

the quantitative determination of BUP. Our preliminary trail was to examine the different compositions of mobile phases consisting of water and methanol on a reversed-phase stationary phase.

Mobile phases consisting of methanol–water were tested at various ratios such as 90:10, 85:15, 80:20, 75:25, 70:30 and 60:40. The variations of mobile phase led to considerable changes in the chromatographic parameters. The optimum mobile phase was methanol–water (75:25) for this system. BUP-NBD derivative appeared at 3.07 min. Several compounds were tested as IS and mexiletine was found to be the most appropriate compound. Mexiletine appeared at 7.60 min and no interference was observed when a certain amount of BUP was spiked into the plasma and urine and extracted from the samples. These are demonstrated in Figure 3.

The derivative of BUP-NBD exhibited morphologically good peaks on the reversed-phase C18 column in the mobile phase when a 1.2 mL/min flow-rate was used. System suitability tests were performed and chromatographic parameters were calculated from experimental data, such as retention time (tr), capacity factor (k'), tailing factor (T), resolution (Rs) and theoretical plate number (N) are given in Table I. All the values for the system suitability parameters are within the acceptable range.

Liquid–liquid extraction

Liquid–liquid extraction was used for the recovery of the sample. Certain organic solvents such as *n*-hexane, ethyl acetate, dichloromethane, chloroform, 2-propanol and their mixtures in different combinations and ratios were tested. A mixture of ethyl acetate was found to be the most suitable solvent to recovery BUP from urine and plasma. Clean chromatograms were obtained when this solvent was used.

Method validation

Precision and accuracy

Precision and accuracy were tested by spiking at three different concentrations (5, 100 and 500 ng/mL for plasma) and (10, 100 and 500 ng/mL for urine). These concentrations are chosen as the lowest, highest and a point chosen randomly. They were entirely in the linear range. BUP was recovered from the samples; the samples were derivatized by utilizing NBD-Cl and injected. The results of the precision (as RSD %)

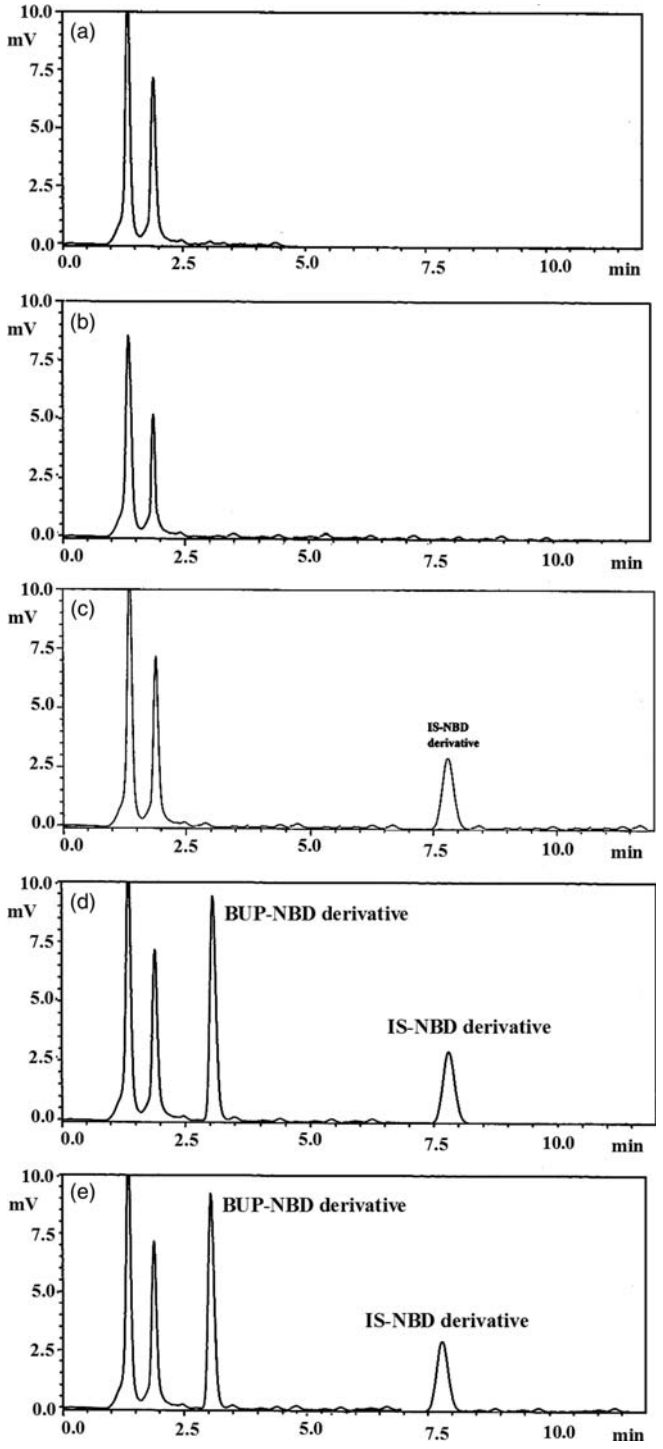

Figure 3. Chromatograms of (a) blank human plasma with NBD-Cl; (b) blank human urine with NBD-Cl; (c) plasma spiked with 100 ng/mL IS-NBD; (d) plasma spiked with 300 ng/mL BUP-NBD; (e) urine spiked with 300 ng/mL BUP-NBD.

and accuracy (as bias) tests of the method in human plasma and urine are listed in Table II.

Linearity

Calibration curve of BUP was linear over the concentration range of 5–500 ng/mL (10, 25, 50, 100, 250 and 500) for plasma and 10–500 ng/mL (5, 25, 50, 100, 250 and 500) for urine. These results are in agreement with those of previously published studies (4–6). Statistical evaluations of the linearity are demonstrated in Table III.

Table I
Results of System Suitability Evaluations

Parameters	IS-NBD	BUP-NBD	Optimum
tR	7.60	3.07	–
k'	4.87	2.31	> 2
T	1.07	1.03	1.10 > T > 0.9
Rs	5.97	2.36	> 2
N	4425	2146	> 2,000

Limits of detection and quantification

The LOD and LOQ values for BUP were found to be between 0.24 and 0.72 ng/mL (as the inter-day results) for plasma and urine. During the linearity studies, the lowest dilution was 5 and 10 ng/mL for plasma and urine, respectively. The values given previously were calculated by utilizing the parameters of linearity, as explained in the Experimental section. These are lower than those obtained by many other reported methods (4–7, 9, 11–12).

Recovery

The recovery of the BUP-NBD associated compound from human plasma, urine and artificial tablet matrix were investigated by standard addition technique. The results (Table IV) showed that the recoveries were in the range of 97.60–99.82%, which is better than those obtained by previously reported methods (4, 9, 11, 12).

Robustness

The robustness of the method was examined by small variations of critical parameters, such as composition of mobile phase and column temperature. The mobile phase was varied ± 1 units, the percentage of methanol in mobile phase was varied $\pm 1\%$ and the column temperature was changed $\pm 2^{\circ}C$. % RSD was ≤ 1.02 and within the limit required for HPLC analysis. It can be concluded that this method is robust, because slight variations of these experimental conditions have little or no effect on the results. According to the relevant results, the method is highly robust.

Specificity

For the formulation samples of BUP, certain excipients such as ethyl cellulose, macrogol, povidone, silicon dioxide and triethyl citrate were checked and no interference was observed with the peaks of BUP and IS. This shows that the method is highly specific.

Stability

Stability of BUP was examined by two ways in plasma and urine. A definite amount of BUP was added into the plasma and urine and they were divided into some tubes. One group was kept in a refrigerator at $-20^{\circ}C$ and another group was kept at ambient temperature. The BUP extract was injected to HPLC and the rPN values were evaluated to decide the stability. BUP was found to be stable at approximately one month in plasma

Table II
Intra-Day and Inter-Day Precision and Accuracy of the Method for the BUP-NBD Compound

	Intra-day (n = 6)									Inter-day (n = 18)		
	First day			Second day			Third day					
	Found ng/mL	RSD%	bias	Found ng/mL	RSD%	bias	Found ng/mL	RSD%	Bias	Found ng/mL	RSD%	Bias
Human plasma	5.2	0.86	+4.0	4.9	0.91	−2.0	5.2	0.86	+4.0	5.1	0.58	+2.0
	100.2	0.18	+0.2	100.3	0.11	+0.3	98.7	0.18	−1.3	99.7	0.16	−0.28
	498.1	0.20	−0.38	498.3	0.21	−0.34	498.6	0.10	−0.28	498.3	0.17	−0.33
Human urine	10.1	0.13	+1.0	9.85	0.82	−1.5	9.87	0.17	−1.3	9.94	0.37	−0.60
	98.3	0.11	−1.7	98.7	0.12	−1.3	98.5	0.16	−1.5	98.5	0.13	−1.50
	497.8	0.17	−0.44	499.0	0.18	−0.2	498.8	0.22	−0.24	498.5	0.19	−0.29

Table III
Statistical Evaluation of Calibration Results for BUP-NBD Derivative by Spiking into Plasma and Urine

	Intra-day (each n = 6)						Inter-day (each n = 18)	
	Plasma			Urine			Plasma	Urine
	First day	Second day	Third day	First day	Second day	Third day		
Range (ng/mL)	5–500			10–500			5–500	10–500
a	0.0016	0.0017	0.0016	0.0016	0.0015	0.0013	0.0017	0.0015
± SD	1.6×10^{-4}	1.5×10^{-4}	1.6×10^{-4}	1.8×10^{-4}	2.0×10^{-4}	2.1×10^{-4}	1.6×10^{-4}	2.0×10^{-4}
b	0.0014	0.0014	0.0015	0.0157	0.0156	0.0156	0.0015	0.0156
± SD	1.26×10^{-4}	1.26×10^{-4}	1.0×10^{-4}	1.2×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.17×10^{-4}	1.1×10^{-4}
r	0.9995	0.9995	0.9996	0.9996	0.9995	0.9995	0.9996	0.9995
LOD	0.26	0.26	0.21	0.25	0.22	0.25	0.24	0.24
LOQ	0.78	0.78	0.63	0.75	0.66	0.75	0.72	0.72

Note: a is slope, b is intercept, r is correlation coefficient, SD is standard deviation of regression equation.

Table IV
Recovery Results of BUP-NBD from Plasma, Urine and Tablet Matrix

Amount of BUP added (ng/Ml)	Amount of BUP found (ng/mL) (mean ± SD, n = 6)	Recovery (%)	RSD (%)
Plasma			
5.0	4.88 ± 0.05	97.60	1.02
100.0	96.15 ± 0.19	96.15	0.19
500.0	488.12 ± 0.50	97.62	0.10
Urine			
10.0	9.52 ± 0.11	95.20	1.15
100.0	96.32 ± 0.14	96.32	0.14
500.0	482.43 ± 0.32	96.48	0.07
Tablet			
2.0	1.97 ± 0.03	98.50	1.52
100.0	98.78 ± 0.55	98.78	0.56
500.0	499.13 ± 0.24	99.82	0.05

Table V
Assay Results of Wellbutrin Tablet Containing 150 mg BUP (n = 6)

Drug	HPLC method	UV spectrophotometric method
	Recovery (%) ± SD	
Quantification of BUP tablet (Wellbutrin Tablet)	$99.87 \pm 7.7 \times 10^{-2}$	$99.76 \pm 9.3 \times 10^{-2}$
t -test of significance a	1.06	
F -test of significance a	1.49	

[a] The tabullatedions of *t* and *F* at 95% confidence limit are 2.23 and 5.05, respectively.

and urine at −20°C and 2 h at ambient temperature. The stability of BUP was also inspected in the mobile phase in the previously described conditions. BUP was kept for 3 h in the mobile phase and 2 h in chloroform at ambient temperature.

Application of the method to a pharmaceutical BUP preparation

The method described here was applied to a pharmaceutical tablet containing 150 mg BUP (Wellbutrin tablet), as described in the Experimental section. The peaks of BUP and IS were identical to those obtained from the standards and no interference from the tablet matrix was exhibited.

The quantification of the tablet was found to be 149.8 mg (HPLC method) and 149.6 mg (comparison method), which corresponded to $99.87 \pm 7.7 \times 10^{-2}$ and $99.76 \pm 9.3 \times 10^{-2}$% BUP ± SD in a tablet. The results were statistically compared with those obtained by the UV spectrophotometric method using *t*- and *F*-tests. No significant difference was found between the two methods in terms of the mean values at the 95% confidence level (Table V). The *t*- and *F*-test significance values were smaller than those of their related tabulated values. Besides, the tablet (Wellbutrin 150 mg) conforms to the requirements for United States Pharmacopeia (XXXI) (29) because the results are in the limits of 90–110%.

Incured sample reanalysis

It was recently recommended that certain samples of BUP in plasma and urine be reanalyzed to ensure method reproducibility, as explained in the Experimental section (19). Assay reproducibility was assessed with pre-defined acceptance criteria requiring that greater than two-thirds of the reanalyzed samples had a relative difference in concentration of ≤20% of

Table VI
Incurred Sample Reanalysis for Plasma And Urine Samples (n = 2 each)

Original concentration (ng/mL)	Reassay concentration (ng/mL)	Mean %	Difference
Plasma			
5.0	5.20	5.10	+3.92
25.0	24.10	24.55	−3.67
50.0	48.10	49.05	−3.87
100.0	100.20	100.10	+0.19
250.0	246.70	248.35	−1.33
500.0	498.20	499.10	−0.36
Urine			
10.0	10.10	10.05	+0.99
25.0	23.60	24.30	−5.76
50.0	48.20	49.10	−3.67
100.0	98.30	99.15	−-1.71
250.0	246.00	248.00	−-1.61
500.0	497.80	498.90	−-0.44

Note: %difference = absolute (reassay value − original value)/average of reassay and original value × 100%.

the original result. The incured sample reanalysis results from 12 samples (six plasma and six urine) are demonstrated in Table VI. All reanalyzed samples gave results that were well within the acceptance criteria, further presenting that this method is capable of producing reproducible results.

Conclusion

This paper describes an HPLC determination of BUP after pre-column derivatization with an NBD-Cl reagent. The proposed method yields reliable analytical results, even at low concentrations of BUP. Compared with existing methods, the described method exhibits some remarkable advantages of the derivatization procedure, such as short analysis time, low derivatization concentration LODs (4–7, 9, 11–12) and low consumption of sample and reagent. Additionally, the recovery percentage of BUP is high (4, 9, 11, 12), the derivatization and extraction processes do not take much time, and additionally, the short retention time is an advantage (9–10). In summary, this paper describes a sensitive and accurate HPLC method for the quantification of BUP in tablet and plasma and urine concentrations during clinical pharmacokinetic studies in humans.

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