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Technical note

Determination of the anti-platelet-activating factor BN-50727 and metabolites in human urine by high-performance liquid chromatography using solid-phase extraction

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Abstract

A sensitive and selective HPLC solid-phase extraction procedure was developed for the determination of platelet-activating factor antagonist BN-50727 and its metabolites in human urine. The procedure consisted in a double solid-phase extraction of the urine samples on cyanopropyl and silica cartridges, followed by an automated solid-phase extraction of the drug and metabolites on CBA cartridges and posterior elution on-line to the chromatographic system for its separation. The method allowed quantitation in the concentration range 10–2400 ng/ml urine for both BN-50727 and the main metabolite, the *O*-demethylated BN-50727 product. The limit of quantitation for both compounds was 10 ng/ml. The inter-assay precision of the method, expressed as relative standard deviation, ranged from 1.9 to 4.5% for BN-50727 and from 2.5 to 9.0% for the metabolite. The accuracy, expressed as relative error, ranged from −2.4 to 4.2% and from 0.2 to 6.2%, respectively. This paper describes the validation of the analytical methodology for the determination of BN-50727 in human urine and also for its metabolites. The method has been used to follow the time course of BN-50727 and its metabolites in human urine after single-dose administration.

Keywords: BN-50727; Platelet-activating factor antagonists

1. Introduction

BN-50727 is a synthetic derivative with potent platelet-activating factor (PAF) antagonizing properties [1,2]. The compound (compound III) is currently under investigations for its anti-allergic, anti-ischemic and anti-inflammatory activities.

Preliminary metabolic studies revealed that compound III is primarily metabolized to the *O*-demethylated BN-50727 product (compound II) in dogs, monkeys, rats and humans [3]. The compound NHPTT (compound I) was also detected in the urine of rats after compound III administration, indicating another possible metabolite of the parent compound.

Recently, a very sensitive method has been published for the determination of compound III in human plasma and urine by combined liquid chromatography–negative ion chemical ionization mass spectrometry [4]. The present paper describes a selective and sensitive HPLC technique using a

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solid-phase extraction (SPE) method for the determination of compounds I, II and III simultaneously. The methodology described here is a complement to that recently described [5] for the determination of the aforementioned compounds in plasma using SPE. Thus, the methodologies to carry out the main pharmacokinetic studies of compound III and metabolites are provided. The method described here has been used widely to follow the human urine levels of BN-50727 and its metabolites after compound III administration at doses of 2.5, 10, 20, 40 and 80 mg.

2. Experimental

2.1. Chemicals and reagents

Compounds I, II, III, the internal standard and most chemicals used in the present study were the same as reported previously [5] (refer to this paper for the structures of compounds I, II, III, and the internal standard).

2.2. Preparation of urine samples

Human urine was obtained from healthy donors, pooled and stored in polypropylene tubes at −80°C until use. After thawing, urine was centrifuged at 2000 × *g* (4°C) for 15 min to separate the clear supernatants in order to prepare the samples. Absence of possible interfering chromatographic peaks was assessed in urine samples from each separate donor before pooling.

2.3. Chromatography, validation protocol and quantitation

Chromatographic separations, validation protocol and quantitation (peak area for all compounds) were carried out according as described previously for the analysis of compounds I, II and III in human plasma [5].

2.4. Solid-phase extraction

SPE of the samples were made on CBA (Analytichem) ion-exchange (propylcarboxylic acid), 10 × 3 mm I.D., disposable cartridges. The cartridges were initially treated with methanol (2 ml), water (2 ml) and ammonium acetate, 20 m*M*, pH 7.0 (2 ml); flow-rate at all steps was 2 ml/min. Urine samples (500 μl) prepared as described below were automatically loaded onto the cartridges which were purged with ammonium acetate, 20 m*M*, pH 7.0 (0.5 ml/min for 2 min) and a mixture of acetonitrile–water 10:90 (2.5 ml/min for 1 min) for cleaning-up. Finally, the cartridges were inserted on-line to the chromatographic system by means of a switching valve in order to elute them with the appropriate solvent gradient programme.

2.5. Standard and sample preparations

Calibration standards were prepared as described previously [5]. Urine calibration curves were performed using urine from healthy donors (blank urine). For this purpose, aliquots of 3.0 ml of blank urine were added to glass tubes containing 2.9 ml of 0.5 *M* sodium phosphate buffer, pH 7.0, and spiked with 75 μl of standard mixture solution containing appropriate amounts of compounds I, II and III, and 25 μl of working internal standard solution. The final concentrations were 10, 20, 50, 100, 300, 600, 1200 and 2400 ng/ml urine for compounds I, II and III, and 75 ng/ml urine for the internal standard.

Urine samples to be analyzed (pharmacokinetic studies) were prepared in the same manner by substituting the addition of the standard mixture solution with the same volume of acetonitrile–water mixture (1:9) solution.

Samples (5.0-ml aliquots) corresponding to the calibration curves and those to be analyzed, prepared as described above, were introduced into Sep-Pak Plus cyanopropyl cartridges. After loading, a first elution step with 5.0 ml ethyl acetate was performed from these cartridges onto Sep-Pak silica cartridges and, in a second step, were eluted from silica cartridges with methanol (5 ml). The methanolic extracts were fully dried under a stream of nitrogen and reconstituted with 2.0 ml acetonitrile–water (1:9) mixture. Finally, the samples thus obtained were introduced in autosampler vials for direct injection of 500 μl through the CBA cartridges as described above.

3. Results and discussion

3.1. Chromatographic separation

The chromatographic profiles obtained from urine blanks corresponding to five different healthy urine donors were very similar. A representative chromatogram is shown in Fig. 1A. A chromatogram corresponding to a urine sample spiked with 300 ng/ml urine of compounds I, II and III and 75 ng/ml urine of the internal standard is shown in Fig. 1B. The comparison between the chromatogram corresponding to the urine blank and the spiked urine showed the absence of interfering peaks at the expected retention times for the compounds of interest.

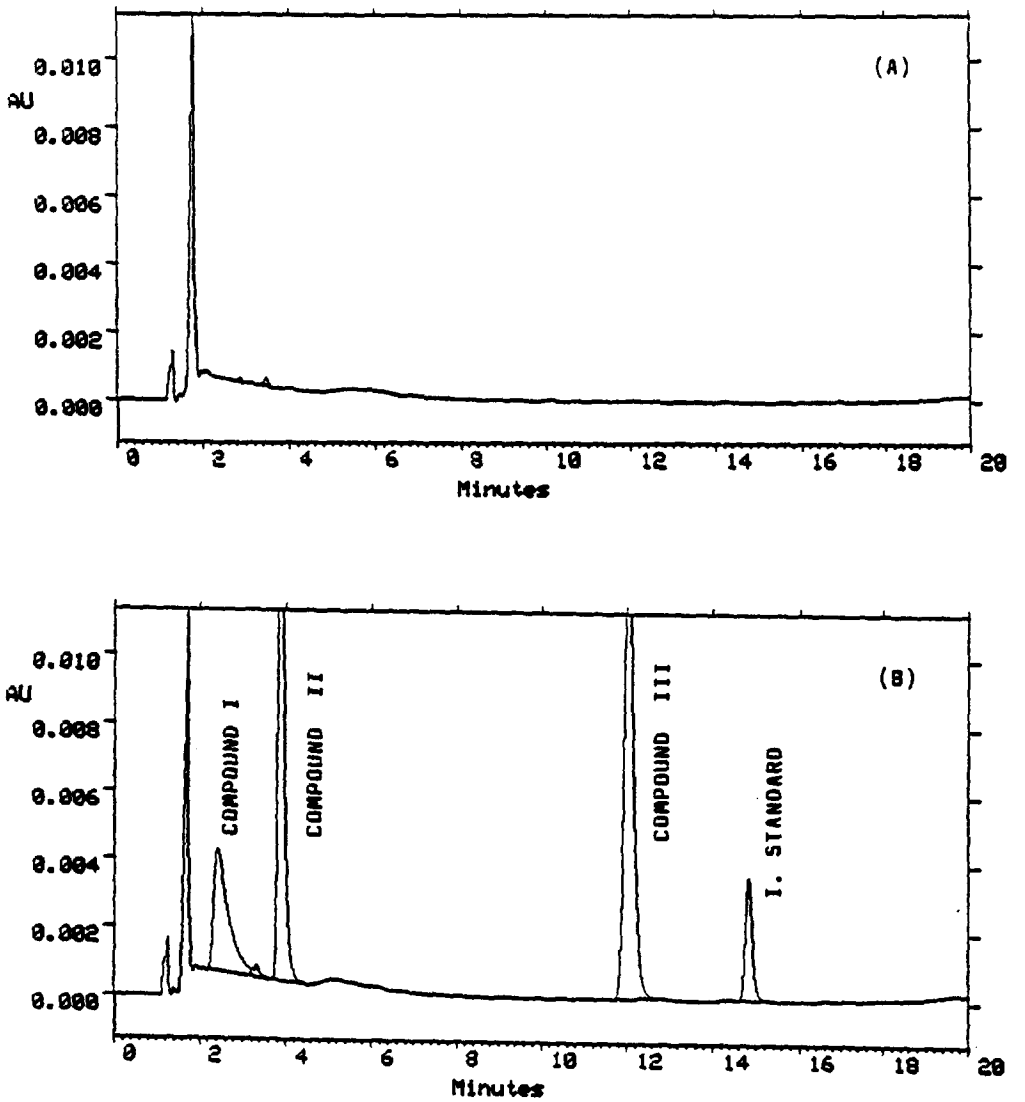

Fig. 1. Chromatograms corresponding to compounds I, II and III in human urine. (A) Urine from a healthy donor (urine blank). (B) Urine from a healthy donor spiked with 300 ng/ml of compounds I, II and III, and 75 ng/ml of the internal standard.

Table 1
Means, standard deviations (S.D.), relative standard deviations (R.S.D.) and relative errors (R.E.) derived from within-run accuracy and precision studies for the determination of compound III and metabolites (compounds I and II) in human urine ($n = 6$)

Effective concentration (ng/ml)	Concentration found (ng/ml)								
	Compound I			Compound II			Compound III		
	Mean ± S.D.	R.S.D. (%)	R.E. (%)	Mean ± S.D.	R.S.D. (%)	R.E. (%)	Mean ± S.D.	R.S.D. (%)	R.E. (%)
10	–	–	–	11.7±1.08	9.2	17.0	10.3±0.55	5.3	3.3
20	–	–	–	19.9±2.20	11.1	−0.5	20.4±0.75	3.7	1.9
50	51.6±2.64	5.1	3.2	52.0±2.49	4.8	4.0	48.9±1.03	2.1	−2.2
100	97.2±6.01	6.2	−2.8	101.7±3.94	3.9	1.7	98.0±1.67	1.7	−2.0
300	326.2±21.90	6.7	8.7	328.4±26.66	8.1	9.5	306.5±6.78	2.2	2.2
600	604.7±41.67	6.9	0.8	611.8±40.64	6.6	2.0	611.2±10.04	1.6	1.9
1200	1049.5±120.72	9.7	4.1	1210.1±48.65	4.0	0.8	1215.0±28.59	2.4	1.3
2400	2114.2±271.50	12.8	−11.9	2065.8±99.35	4.8	−13.9	2335.9±44.63	1.9	−2.7

3.2. Precision and accuracy

The results obtained for six-fold replicate samples prepared in a control matrix and analyzed against a duplicate calibration line provided the within-run (intra-assay) precision and accuracy data. These results are shown in Table 1.

The results obtained for two-fold replicate samples prepared in a control matrix and analyzed on four different days against duplicate calibration lines provided the between-run (inter-assay) precision and accuracy data. These results are shown in Table 2.

3.3. Limit of quantitation

The limit of quantitation (LOQ) was defined as the concentration of compound that produces a peak area equal to the mean blank plus three standard deviations, at least, with acceptable accuracy and precision (20%). This was fixed at 50 ng/ml urine for compound I and 10 ng/ml for compounds II and III.

3.4. Linearity

Linearity for compounds I, II and III was checked in the 10–2400 ng/ml urine concentration range.

Table 2
Means, standard deviations (S.D.), relative standard deviations (R.S.D.) and relative errors (R.E.) derived from between-day accuracy and precision studies for the determination of compound III and metabolites (compounds I and II) in human urine ($n = 6$)

Effective concentration (ng/ml)	Concentration found (ng/ml)								
	Compound I			Compound II			Compound III		
	Mean ± S.D.	R.S.D. (%)	R.E. (%)	Mean ± S.D.	R.S.D. (%)	R.E. (%)	Mean ± S.D.	R.S.D. (%)	R.E. (%)
10	–	–	–	10.1±0.61	6.1	1.0	10.1±0.46	4.5	0.7
20	–	–	–	20.3±1.83	9.0	1.4	19.7±0.85	4.3	−1.3
50	48.1±5.93	12.3	−3.7	52.4±3.81	7.3	4.8	48.8±1.79	3.7	−2.4
100	103.0±14.29	13.9	3.0	104.3±7.72	7.4	4.3	99.4±1.91	1.9	−0.6
300	314.2±24.55	7.8	4.7	310.1±18.62	6.0	3.4	309.0±7.45	2.4	3.0
600	556.4±65.79	11.8	−7.3	608.0±45.83	7.5	1.3	612.8±18.85	3.1	2.1
1200	1167.9±84.48	7.2	−2.7	1202.0±29.86	2.5	0.2	1217.7±23.72	1.9	1.5
2400	2393.9±282.37	11.8	−0.3	2548.5±96.30	3.8	6.2	2501.8±60.72	2.4	4.2

The analysis of linearity was made as described previously [5].

For compound I, linearity could be demonstrated between 50 and 2400 ng/ml concentration range and for compound II between 10 and 2400 ng/ml. For compound III, significant differences ($P < 0.05$) in the analysis of variance of the normalized response ratio were obtained at the 10 ng/ml concentration level. However, the precision (1.8%) and accuracy (1.4%) of back-calculated values (data not shown) from the calibration lines for this compound at 10 ng/ml made the quantitation useful at this low concentration level.

The coefficients of regression corresponding to the calibration lines involved during the validation study, expressed as mean ± S.D. values, were 0.9971 ± 0.00173 for compound I, 0.9975 ± 0.00166 for compound II and 0.9993 ± 0.00036 for compound III ($n = 5$).

3.5. Recovery

The recovery of the analytical method was studied at all the concentration levels used in the calibration curves. The application of one-way analysis of variance to the absolute recovery values obtained in 4 days of analysis did not show significant differences ($P > 0.05$) among the concentration levels for each compound. Therefore, the recovery remained constant across the concentration ranges studied being around 32.7 ± 1.78, 53.2 ± 2.39 and $64.4 \pm 3.33\%$ for compounds I, II and III, respectively.

Recovery for the internal standard, studied only at the working concentration, was $56.2 \pm 1.93\%$.

The methodology described here has been used for pharmacokinetic studies in humans, and provided enough sensitivity to follow thoroughly the time course of compound III and its metabolites in urine after the administrations of 2.5-, 10-, 20-, 40- and 80-mg doses. The accuracy of quality control samples (QC) for compounds I, II and III, obtained along different pharmacokinetic studies ($n = 38$), were better than 1.1%, 5.2% and 5.0%, respectively. Precision, on the other hand, expressed as R.S.D., were lower than 11.3%, 9.5% and 10.6%, respectively, for compounds I, II and III (the concentrations of QC samples used were 25, 100 and 400 ng/ml). These values of accuracy and precision for QC samples indicate acceptable values for the analytical technique.

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