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Study of the solid-phase extraction of diclofenac sodium, indomethacin and phenylbutazone for their analysis in human urine by liquid chromatography

A. Bakkali, E. Corta, L.A. Berrueta*, B. Gallo, F. Vicente

Department of Analytical Chemistry, Faculty of Sciences, University of the Basque Country, P.O. Box 644, E-48080 Bilbao, Spain

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Abstract

A selective semi-automated solid-phase extraction (SPE) of the non-steroidal anti-inflammatory drugs diclofenac sodium, indomethacin and phenylbutazone from urine prior to high-performance liquid chromatography was investigated. The drugs were recovered from urine buffered at pH 5.0 using C_{18} Bond-Elut cartridges as solid sorbent material and mixtures of methanol–aqueous buffer or acetonitrile–aqueous buffer as washing and elution solvents. The extracts were chromatographed on a reversed-phase ODS column using 10 m*M* acetate buffer (pH 4.0)–acetonitrile (58:42, v/v) as the mobile phase, and the effluent from the column was monitored at 210 nm with ultraviolet detection. Absolute recoveries of the anti-inflammatory drugs within the range 0.02–1.0 μg/ml were about 85% for diclofenac and indomethacin, and 50% for phenylbutazone without any interference from endogenous compounds of the urine. The within-day and between-day repeatabilities were in all cases less than 5% and 10%, respectively. Limits of detection were 0.007 μg/ml for diclofenac sodium and indomethacin and 0.035 μg/ml for phenylbutazone, whereas limits of quantitation were 0.02 μg/ml for diclofenac and indomethacin and 0.1 μg/ml for phenylbutazone.

Keywords: Diclofenac sodium; Indomethacin; Phenylbutazone

1. Introduction

When traces of drugs must be determined in complex matrices such as biological fluids, a sample handling procedure is usually needed prior to the chromatographic analysis. The aims of the sample pre-treatment are the release of the analyte from a conjugate (chemical or enzymatic hydrolysis) or from proteins in the biological matrix, the elimination of proteins, which can clog the chromatography column, and of endogenous compounds that can interfere in the assay (sample clean-up) and an increase in the concentration of the analyte to reach the detection range of the detector (trace enrichment).

Different methods of sample preparation such as deproteinization, ultrafiltration, addition of a proteolytic enzyme, dilution, liquid–liquid extraction (LLE) and liquid–solid extraction (LSE) or solid-phase extraction (SPE) can be used. When they are performed manually, these sample preparation techniques are often tedious and time-consuming. Therefore, techniques with high automation potential, such as SPE, are of particular interest when the number of

*Corresponding author. Fax: +34-94-4648-500.

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samples is relatively large. In addition, the development of an automated sample handling procedure often leads to better results with respect to accuracy and precision.

Non-steroidal anti-inflammatory drugs (NSAIDs) with analgesic and antipyretic properties are widely used as the first-choice agents in the treatment of patients with rheumatoid arthritis and osteoarthritis [1,2]. Many of the analytical methods for these substances in biological fluids are based on high-performance liquid chromatography (HPLC) systems [3–25], most of them using direct injection of urine or LLE treatment of plasma or urine. Only in few cases SPE is used [22–25], but the SPE process was not exhaustively optimized.

This paper deals with the optimization of all the experimental variables that affect the recoveries obtained in SPE, not only physico–chemical variables such as the nature of the solid and liquid phases used and the sample pH, but also other hydrodynamic variables as the flow-rate at which liquid phases are passed through the adsorbent or the volume of air used to force the liquids through the solid. Three typically used NSAIDs were chosen as test compounds.

2. Experimental

2.1. Reagents and apparatus

Diclofenac sodium and phenylbutazone were kindly supplied by Geigy (Barcelona, Spain) and indomethacin by Alfarma (Barcelona, Spain) and the internal standard, *p*-hydroxybenzoic acid *n*-butyl ester (butyl paraben) was from Sigma (Madrid, Spain). Methanol and acetonitrile (Romil, Barcelona, Spain) were of HPLC grade. The water used in all experiments was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Sodium acetate, acetic acid, phosphoric acid, sodium hydrogen and dihydrogen phosphates, boric acid, sodium borate and sodium hydroxide were of analytical quality from Merck (Darmstdt, Germany). Aqueous buffers were prepared to buffer the aqueous standard and spiked urine samples. The buffers were: H_3PO_4–NaH_2PO_4 (pH 2.0), HAc–NaAc (pH 3.5 to 5.5), NaH_2PO_4–Na_2HPO_4 (pH 6.0 to 8.0), H_3BO_3–NaH_2BO_3 (pH 9.0 to 10). Stock standard solutions of diclofenac sodium, indomethacin and phenylbutazone were prepared in methanol at a concentration of 1 mg/ml and were stored at 4°C in the dark.

The SPE cartridges used were Bond-Elut (Scharlau, Barcelona, Spain) octadecyl (C_{18}) silica bonded phase (100 mg). The extractions were performed with an ASPEC (automatic sample preparation with extraction cartridges) XL equipment from Gilson (Villiers le Bel, France). The extracts were evaporated to dryness using a Zymark (Barcelona, Spain) Turbo Vap LV evaporator. No recovery or selectivity problems were observed when the same cartridge was used up to five times for urine samples and 15 times with aqueous standard samples.

HPLC was used for the determination of the recoveries of the drugs eluted from the cartridges. The chromatographic system consisted on a LKB (Barcelona, Spain) 2248 pump, a Hewlett-Packard (Barcelona, Spain) 1100 automatic sample injector with a loop of 100 μl and a Waters (Barcelona, Spain) 484 UV–Visible detector. A reversed-phase Waters Nova-Pack C_{18} column (15 cm×3.9 mm I.D., 4 μm particle size) and a Waters Nova-Pack C_{18} guard-column (20 mm×3.9 mm I.D., 4 μm particle size) were used. The effluent was monitored at 210 nm. The mobile phase was acetonitrile–10 m*M* acetate buffer (pH 4.0) (42:58, v/v) delivered at a flow-rate of 1 ml/min and the volume injected was 20 μl. The system was operated at room temperature.

2.2. Extraction procedure

2.2.1. SPE studies in aqueous standard samples

Buffered aqueous samples of 0.5 μg/ml were prepared by dilution from stock solutions using the appropriate 0.1 *M* aqueous buffer. The extraction was performed by the ASPEC system in a sequential mode. First, the SPE cartridge was activated with 2 ml of methanol and was washed with 1 ml of aqueous buffer. After that, 1 ml of aqueous standard sample was added and the compounds of interest were eluted with 1 ml of the adequate elution solvent.

For the pH study, the elution solvent was methanol and for the elution study (recoveries evaluated as a function of the composition of the elution solvent)

Table 1
Recoveries [%, ±relative standard deviation (RSD) of $n=4$ determinations] of the analytes from aqueous standard samples using C_{18} cartridges as a function of the sample pH (elution solvent: methanol)

	pH						
	2.0	3.0	4.0	5.0	5.5	6.0	7.0
Diclofenac Na	96.6±2.9	100.79±0.98	100.67±0.92	102.6±2.7	103.6±3.6	102.5±3.6	89.9±3.1
Indomethacin	71.7±1.9	81.9±3.3	90.1±1.2	100.3±2.8	102.0±2.0	96.7±2.9	86.2±1.3
Phenylbutazone	96.8±1.8	93.7±1.2	102.6±1.1	101.35±0.37	102.4±5.0	102.8±3.1	

methanol–aqueous buffer mixtures and acetonitrile–aqueous buffer mixtures were examined.

2.2.2. SPE studies in spiked urine samples

Blank urine samples were spiked with 0.5 μg each of diclofenac sodium and indomethacin and 1 μg of phenylbutazone per ml of urine and buffered with the appropriate 1.0 *M* buffer (urine–aqueous buffer, 5:1, v/v). The SPE procedure was similar to the case of the aqueous standard samples, but the cartridge was washed with 1 ml of the convenient washing solvent prior to the elution with 1 ml of the adequate elution solvent.

The extracts from all studies were evaporated to dryness under a nitrogen stream at 45°C and reconstituted with 200 μl of mobile phase containing 5 μg/ml of internal standard. A 20-μl volume of this solution was injected into the chromatographic system. Addition of internal standard was performed after the SPE process in order to only correct errors in the reconstitution, injection, separation and detection steps.

3. Results and discussion

First of all, SPE from aqueous standard samples was investigated using the most apolar sorbent (C_{18}). The recoveries obtained in the study of the effect of sample pH are presented in Table 1 and show that high recoveries are reached for the three drugs over the pH range 2.0–7.0, obtaining complete recoveries at pH 5.0–5.5. For basic pH values losses on recoveries of diclofenac sodium and indomethacin and a possible degradation of phenylbutazone were observed. Then, recoveries were evaluated as a function of the composition of the elution mixture (methanol–aqueous buffer or acetonitrile–aqueous buffer), fixing the sample pH to 5.0 and the results are shown in Table 2. The plot of the recovery versus the organic percentage of the elution solvents gives information about the best composition of the washing solvent (the one with the highest organic solvent percentage without elution of the drug) and the best elution solvent (the lowest organic solvent percentage that gives the complete recovery of the

Table 2
Recoveries [%, ±relative standard deviation (RSD) of $n=4$ determinations] of the analytes from aqueous standard samples at pH 5.0 using C_{18} cartridges as a function of the composition of the elution mixture (A: methanol–aqueous buffer, pH 5.0 and B: acetonitrile–aqueous buffer, pH 5.0)

A	% MeOH								
	40	50	60	70	80	90	100		
Diclofenac Na	<0.1	10.53±0.88	74.83±0.90	99.6±3.2	96.9±1.9	97.9±2.7	99.8±3.1		
Indomethacin	<0.1	1.66±0.17	45.2±1.1	92.2±1.7	90.8±2.2	91.0±1.8	93.4±1.0		
Phenylbutazone	<0.5	24.7±1.2	93.3±1.3	100.8±3.7	98.8±1.8	100.5±2.6	101.2±2.1		
B	% CH_3CN								
	20	30	40	50	60	70	80	90	100
Diclofenac Na	<0.1	7.3±5.2	87.6±1.2	95.4±1.1	93.8±1.8	90.3±2.0	89.7±2.5	89.3±1.0	92.6±1.0
Indomethacin	<0.1	5.2±4.6	69.5±2.3	97.9±1.0	93.4±2.3	92.2±1.1	92.5±1.0	94.2±1.3	92.3±2.1
Phenylbutazone	<0.5	2.0±7.4	43.5±3.5	97.0±1.1	93.3±2.5	92.5±2.1	93.0±3.1	88.7±2.0	87.6±1.0

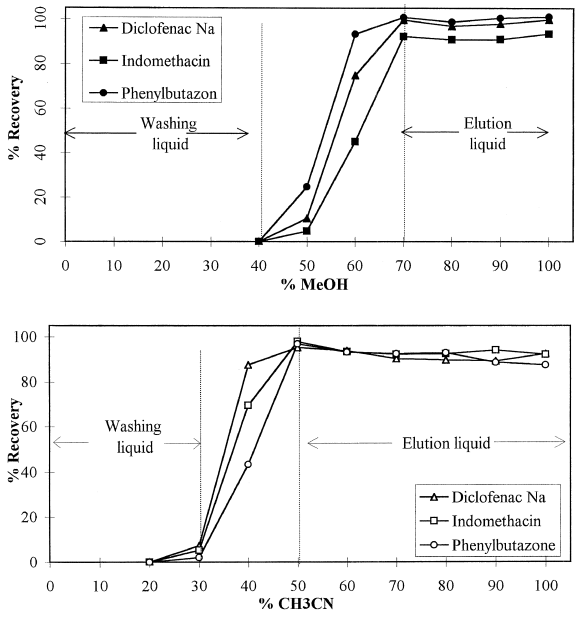

Fig. 1. Plot of the recoveries of the drugs from aqueous standard samples at pH 5.0 using C_{18} cartridges versus the composition of the elution mixture (methanol–aqueous buffer or acetonitrile–aqueous buffer).

drug) (Fig. 1). The correct selection of the washing and elution solvents will provide the cleanest samples in the SPE process and therefore the best selectivity in the extraction.

Thus, the optimum conditions found in the aqueous standard samples study, using the apolar cartridge C_{18}, for the three drugs were 1 ml of methanol–acetate buffer (40:60, v/v) as the washing solvent and 1 ml of methanol–acetate buffer (70:30, v/v) as the elution solvent or 1 ml of acetonitrile–acetate buffer (30:70, v/v) as the washing solvent and 1 ml of acetonitrile–acetate buffer (50:50, v/v) as the elution solvent fixing the pH of the samples to 5.0 in both cases.

The selectivity of the extraction was checked using urine samples spiked with 1 μg of phenylbutazone and 0.5 μg both of diclofenac sodium and indomethacin per ml. The best selectivity and recovery were attained when acetonitrile was used in the washing and elution mixtures: 84.7±4.0, 81.2±2.5 and 50.9±2.3% (n=4) in the case of CH_3CN versus 78.8±3.8, 83.4±2.0 and 45.2±2.1% (n=4) in the case of CH_3OH, for diclofenac, indomethacin and phenylbutazone, respectively.

Other experimental variables related with the hydrodynamic performance of the ASPEC equipment, were also tested using spiked urine samples and acetonitrile as organic modifier of the washing and elution solvents. The three major variables are the air volume passed through the cartridge after the sample loading and after the washing steps (inadequate drying of SPE column can cause losses in recovery), the volume of elution solvent used and the flow-rate at which the elution solvent is passed through the cartridge. The effect on recoveries of the air volume used after the loading process is shown in Fig. 2A, where losses in recoveries of the three drugs can be observed for air volumes lower than 0.5 ml

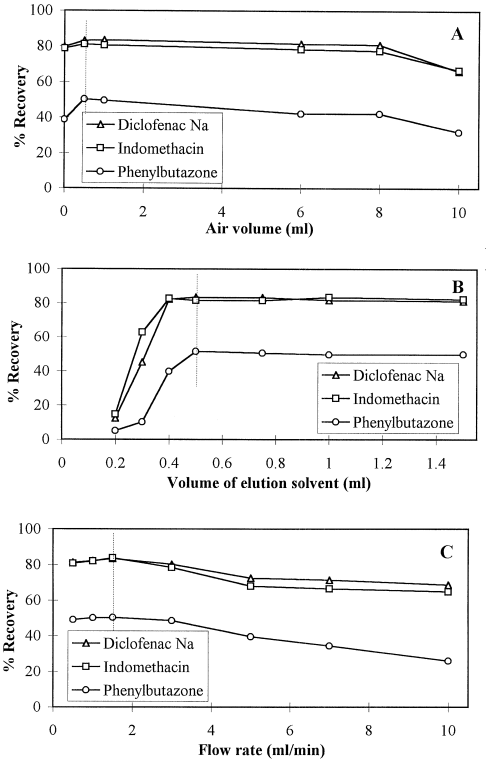

Fig. 2. Effects on recoveries of air volume passed through the SPE column after the sample loading step (A), volume of elution solvent (B) and flow-rate of the elution process (C).

and higher than 1 ml (specially with phenylbutazone). No significant decreases in recoveries were observed in the case of the air volume passed after the washing step (volumes between 0 and 10 ml were tested). Fig. 2B and C shows losses in recoveries when volumes of elution solvent lower than 0.5 ml and flow-rate of the elution process higher than 1.5 ml/min were used.

Thus, the optimum conditions finally proposed for the SPE of the tested compounds in urine samples were (total cycle: 10.5 min.): (i) cartridge conditioning (flow-rate: 6.0 ml/min; air volume: 0.3 ml): the cartridge (C_{18}) was first conditioned with 2.0 ml of methanol and then with 1.0 ml of 0.1 *M* acetate buffer (pH 5.0). (ii) Loading with sample (flow-rate: 1.50 ml/min; air volume: 0.5 ml): 1 ml of sample buffered with 1 *M* acetate buffer (pH 5.0) was dispensed on the cartridge. (iii) Washing (flow-rate: 3.0 ml/min; air volume: 1.5 ml): the cartridge was washed with 1 ml of acetonitrile–0.1 *M* acetate buffer (pH 5.0) (30:70, v/v). (iv) Elution (flow-rate: 1.5 ml/min; air volume: 1.5 ml): 0.5 ml volume of acetonitrile–0.1 *M* acetate buffer (pH 5.0) (50:50, v/v). was applied to the cartridge.

Using the C_{18} cartridges and these optimum extraction conditions, a validation study was carried out, testing the selectivity, linearity, absolute recovery, repeatability and limits of detection and quantitation of the assay.

Typical chromatograms of a blank urine extract and a spiked urine extract containing diclofenac sodium, indomethacin and phenylbutazone are given in Fig. 3. The extract of drug-free urine shows the absence of endogenous peaks from the urine matrix at the retention times corresponding to analytes.

Urine samples spiked with six different concentrations of the drugs were analyzed using the optimum SPE condition in quintuplicate. The peak area ratios (analyte-to-internal standard) showed a linear relationship with the concentration over the range 0.02–1.0 μg/ml for diclofenac sodium and indomethacin and 0.1–1.0 μg/ml for phenylbutazone. The equations obtained using the least-squares method were $y=(0.8109\pm0.0051)x+(0.0034\pm0.0024)$ ($r^2=0.9989\pm0.0097$) for diclofenac sodium, $y=(0.8110\pm0.0070)x+(0.0039\pm0.0033)$ ($r^2=0.998\pm0.013$) for indomethacin and $y=(0.513\pm0.014)x-(0.0042\pm0.0082)$ ($r^2=0.99\pm0.22$) for phenylbutazone, where y is the peak-area ratio and x is the drug concentration in urine samples (μg/ml).

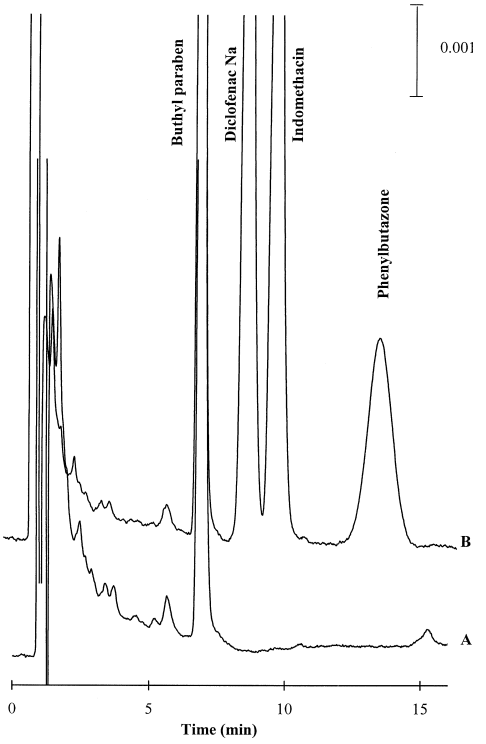

Fig. 3. Typical chromatograms obtained by coupling the semi-automated SPE sample preparation to HPLC: (A) blank of urine and (B) urine sample spiked with 1 μg each of diclofenac Na, indomethacin and phenylbutazone per ml of urine, respectively.

The absolute recoveries of the analytes at different concentration levels ranging from 0.02 to 1.0 μg/ml were examined. The mean absolute recoveries for the three analytes were around 85% for diclofenac and indomethacin and 50% for phenylbutazone (Table 3). The precision of the bioanalytical method, expressed by the relative standard deviation (RSD), was estimated by measuring the within-day and between-day repeatabilities at different concentration levels ranging from 0.02–1.0 μg/ml (Table 4). The intra-day repeatabilities ($n=5$) were less than 3% for diclofenac sodium, 4% for indomethacin and 5% for phenylbutazone, and the inter-day repeatabilities ($n=$

Table 3
Absolute recoveries for the determination of drugs in human urine ($n=5$)

Compound	Amount added (μg/ml)	Recovery (%) (mean±SD)
Diclofenac Na	0.02	83.5±2.7
	0.05	86.6±2.6
	0.1	82.32±0.87
	0.25	85.6±2.0
	0.5	86.3±1.2
	1	82.7±5.0
Indomethacin	0.02	85.4±4.1
	0.05	81.2±6.0
	0.1	89.0 ±3.7
	0.25	87.6±1.5
	0.5	81.9±5.6
	1	82.2±4.5
Phenylbutazone	0.1	50.3±1.6
	0.25	50.8±4.8
	0.5	47.8±2.8
	1	48.2±2.5

3) were less than 10% for diclofenac sodium, 9% for indomethacin and 6% for phenylbutazone.

The limits of detection (LODs), calculated as the spiked analyte concentration that produces a chromatographic peak whose height is three-times the baseline noise of chromatograms of blank urine samples were 0.007 μg/ml for diclofenac sodium and indomethacin and 0.035 μg/ml for phenylbutazone.

The limits of quantitation (LOQs), defined as the lowest concentration that produces a chromatographic peak whose height is ten-times the baseline noise of chromatograms of blank urine samples were 0.02 μg/ml for diclofenac and indomethacin and 0.1 μg/ml for phenylbutazone.

Finally, the method was applied to urine samples from healthy subjects following the intake of an oral dose of 100 mg of diclofenac sodium (urine sample collected during the period of 8–14 h after intake) (Fig. 4A), 50 mg of indomethacin (sample 0–14 h) (Fig. 4B) and 100 mg of phenylbutazone (sample 12–14 h) (Fig. 4C). No interferences from metabolites of the drugs were observed.

4. Conclusion

An easy and quick bioanalytical method for the assay of three NSAIDs (diclofenac sodium, indomethacin and phenylbutazone) based on a semi-automated SPE and reversed-phase HPLC with UV detection have been developed. Optimal selectivities and recoveries were obtained by means of the study of multiple variables that affect the performance of the SPE processes.

The complete analysis time was about 27 min: 10.5 min for the SPE treatment, 1.7 min for the extract evaporation (17 min, 10 samples simultaneously) and 15 min for the chromatographic separation.

If sensitivity is not critical the evaporation step could be avoided and direct injection of the extracts

Table 4
Within-day ($n=5$) and between-day ($n=3$) repeatabilities of the SPE–HPLC determination method

Amount added (μg/ml)	Diclofenac sodium		Indomethacin		Phenylbutazone	
	Amount found (mean±SD) (μg/ml)	RSD (%)	Amount found (mean±SD) (μg/ml)	RSD (%)	Amount found (mean±SD) (μg/ml)	RSD (%)
Intra-day repeatability ($n=5$)						
0.02	0.01703±0.00045	2.6	0.01727±0.00023	1.3	–	–
0.1	0.08343±0.00028	0.34	0.08495±0.00012	0.14	0.0503±0.0022	4.4
0.25	0.2146±0.0032	1.5	0.2192±0.0037	1.7	0.1314±0.0064	4.9
1	0.836±0.012	1.4	0.843±0.028	3.3	0.499±0.015	3.0
Inter-day repeatability ($n=3$)						
0.02	0.01745±0.00050	2.9	0.01722±0.00081	4.7	–	–
0.1	0.08231±0.00078	0.95	0.08874±0.00055	0.62	0.0457±0.0019	4.2
0.25	0.2157±0.0031	1.4	0.205± 0.014	6.8	0.1235±0.0065	5.3
1	0.807±0.079	9.8	0.782±0.068	8.7	0.4741±0.0070	1.5

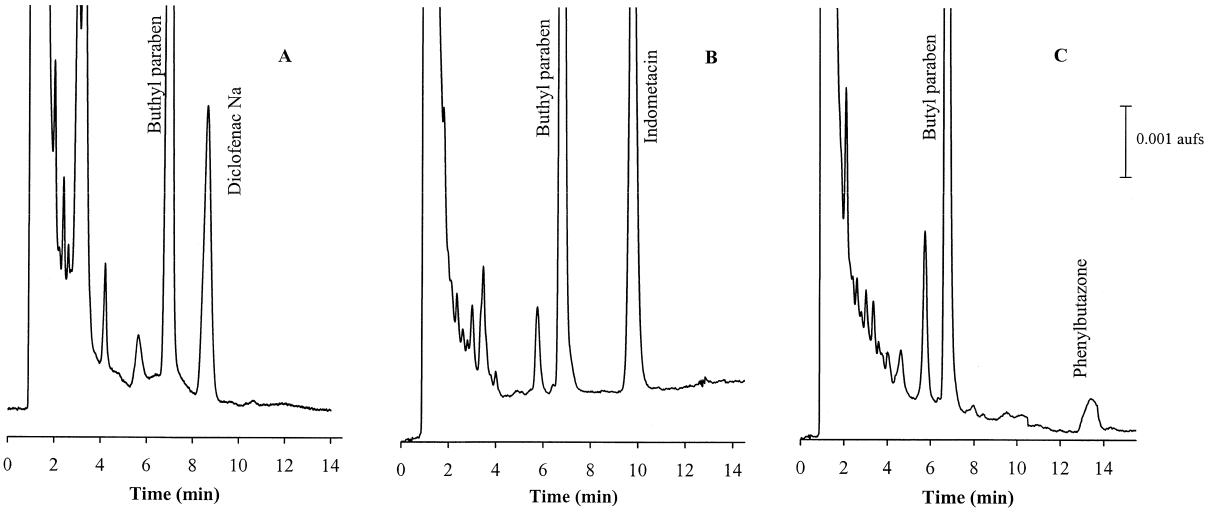

Fig. 4. Chromatograms corresponding to urine samples from three healthy volunteers following the intake of an oral dose of: (A) 100 mg of diclofenac sodium (urine collected between 8–14 h of the intake); (B) 50 mg of indomethacin (urine collected between 0–10 h of the intake); (C) 100 mg of phenylbutazone (urine collected between 12–14 h of the intake).

could be performed reducing the analysis time. In this case complete on-line automation of the SPE and reversed-phase HPLC–UV processes could be accomplished with the ASPEC XL equipment.

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