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Solid-phase extraction of prostanoids using an automatic sample preparation system

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ABSTRACT

A commercial automated solid-phase extraction system for cyclooxygenase arachidonic acid metabolites in urine samples has been evaluated. Comparison of manual and automatic batch (36 samples) extraction procedures for tritium labelled prostanoids added as tracers to urine samples has shown equivalent results with recoveries greater than 90% for prostaglandins E_2, $F_{2\alpha}$ and 6-keto prostaglandin $F_{1\alpha}$ as well as thromboxane B_2. Analyte stability is not affected by the automated procedure, which uses less solvents and has a faster overall processing time than the manual method. The automated system has been applied to the extraction of prostanoids in urine samples from workers exposed to dichloroethane.

INTRODUCTION

Prostaglandins (PGs), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) are cyclooxygenase metabolites of arachidonic acid (AA), collectively known by the generic term prostanoids. Current evidence supports the potential pathophysiological role played by prostanoids in many diseases [1]. The prostanoid pathway can be studied *in vivo* by determining its metabolites in biological fluids [2]. Thus determination of the AA metabolites 6-keto-$PGF_{1\alpha}$ (from PGI_2), TXB_2 (from TXA_2), $PGF_{2\alpha}$ and PGE_2 in urine has been shown to be a powerful way to estimate changes in the total body production of prostanoids [3–5].

One of the problems encountered in the determination of these compounds is to quantify them at their occasionally extremely low physiological concentrations. Suitable analytical procedures for prostanoids include solid reversed-phase extraction, purification by high-performance liquid chromatography (HPLC) and determination of selected HPLC fractions by radioimmunoassay (RIA) [6]. Reversed solid-phase extraction has an important limitation, namely it requires several time-consuming steps [7].

Commercial systems that completely automate solid-phase extraction have become available. One of these systems, ASPEC (automatic sample preparation with extraction columns), has been evaluated in this laboratory as a possible automated extraction method for determining cyclooxygenase AA metabolites in urine. For this purpose the extraction recoveries obtained from urine samples supplemented with tritiated standards of 6-keto-$PGF_{1\alpha}$, TXB_2, $PGF_{2\alpha}$ and PGE_2 have been determined using both the manual and ASPEC extraction protocols. To establish the chemical stability of these prostanoids during extraction HPLC radiochromatographic profiles of urine samples extracted by ASPEC and those extracted manually have been compared [8]. This method provides excellent recoveries for cyclooxygenase AA metabolites with low intra-assay variation and the same radiochromatographic profiles as those obtained manually. Finally, using this method prostanoids were determined in urine samples from workers exposed to dichloroethane and in control subjects.

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EXPERIMENTAL

Apparatus

The ASPEC from Gilson (Villiers-le-Bel, France) consisted of three modules: a sample processor and injector module with a 143 mm stroke vertical arm and a 10-ml syringe for the dilutor, one Model 401 dilutor in slave configuration with its specific standard accessories (dilutor pipetting valve) and an automatically controlled module of various kinds of sample racks.

Air pressure was used to force the samples and eluents through the extraction columns. The HPLC system consists of two ABI 400 pumps from Applied Biosystems (Ramsey, NJ, USA), a high-pressure dynamic mixer, a 100 S diode array detector from Applied Biosystems to control both pumps and a radioactivity detector (Ramona) from Issomes (Straubenhardt, Germany) or a 2211 Superrack collector (LKB, Bromma, Sweden).

Cartridges and HPLC columns

Extractions were carried out on C_{18} cartridges (Amprep C_{18}) (400 mg adsorbent, 40 μm mean particle size) purchased from Amersham International (Buckinghamshire, UK) and on Amprep C_{18} minicolumns (100 mg adsorbent, 40 μm mean particle size). Reversed-phase HPLC was carried out on a Spherisorb ODS-2 column (25 cm × 4.6 mm I.D.; particle size 10 μm) from Phase Separations (Deesire, UK).

Chemicals

Tritiated 6-keto-$PGF_{1\alpha}$ (180 Ci/mmol), TXB_2 (120 Ci/mmol), $PGF_{2\alpha}$ (180 Ci/mmol) and PGE_2 (160 Ci/mmol) were from Amersham International. Methanol, acetonitrile and isopropanol were from Merck (Darmstadt, Germany). Petroleum ether and methyl formate were from Fluka (Buchs, Switzerland). TXB_2 and PGE_2 antisera were provided by the Institute Pasteur (Marnes la Coquette, France).

Samples

Urine samples from workers exposed to dichloroethane and from corresponding matched control subjects were stored at −40°C until required.

Manual procedure

Urine samples (5 ml) were spiked with [^{3}H]6-keto-$PGF_{1\alpha}$, [^{3}H]TXB_2, [^{3}H]$PGF_{2\alpha}$ and [^{3}H]PGE_2 (90 000 dpm/ml of each tritiated prostanoid). Spiked samples were acidified at pH 3.15 with 1 *M* HCl and centrifuged at 1500 *g* for 10 min at 4°C. Aliquots of the supernatants were then processed through C_{18} Amprep columns previously activated with methanol and acidified water (pH 3.15). After passing the urine samples through the columns they were washed with acidified water and petroleum ether. Finally the prostanoids were eluted with methyl formate, which was vacuum-evaporated in a concentrator-evaporator from Savant Instruments (Hicksville, NY, USA) [9] (see Table I).

ASPEC procedure

Aliquots of prostanoid-spiked urine samples, centrifuged and acidified as described above were automatically extracted on the ASPEC system. Briefly, 1 ml of urine was processed through C_{18} Amprep cartridges (100 mg adsorbent) previously activated with 2 ml of methanol and 2 ml of acidified water (pH 3.15). After washing the column with 2 ml of water and 2 ml of light petroleum (b.p. 40–60°C) prostanoids were eluted with 3 ml of methyl formate which was then vacuum-evaporated (see Table I).

TABLE I

COMPARISON BETWEEN MANUAL AND AUTOMATED (ASPEC) EXTRACTION OF PROSTANOIDS IN URINE SAMPLES

	Extraction method		
	Manual		ASPEC
Adsorbant (mg)			
C_{18}	400	100	100
Activation (ml)			
Methanol	10	2	2
Water	10	2	2
Sample volume (ml)			
Urine	5	1	1
Washing solvent (ml)			
Water	10	2	2
Light petroleum ether	20	2	2
Elution (ml)			
Methyl formate	8	3	3

HPLC procedure

The dry residue was redisolved in the mobile phase [40 m*M* formic acid, pH 3.15, with triethylamine–acetonitrile (65:35, v/v) at flow-rate of 1.5 ml/min] and processed through the HPLC system [10]. The HPLC system was connected either to a radioactivity detector or to a fraction collector. The later was used to obtain purified fractions for subsequent RIA determination as described in detail elsewhere [6].

RESULTS AND DISCUSSION

The analytical procedures used in the manual and the automated solid-phase extraction determinations of urinary prostanoids are summarized in Table I. As the volumetric characteristics of the system do not allow the use of eluent volumes greater than 3 ml, the routine manual extraction procedure was scaled down to make the adsorbent bed (100 mg) and solvent eluent volumes directly comparable with those used in the ASPEC system. This resulted in an overall reduction in the analysis time as well as lower solvent consumption in the automated batch processing mode for a total of 36 samples.

Table II shows the recoveries obtained in the manual and automated extraction procedures. The samples used were human urine samples spiked with radioactively-labelled prostanoid standards. The recoveries are equivalent for all practical purposes and are greater than 90% when the smaller (100 mg) cartridges are used in the ASPEC system. The intra-assay variation of the ASPEC procedure equivalent or better than that of the manual procedure.

The data in Table III show the results obtained after extraction and HPLC separation of human urine samples spiked with tritium-labelled prostanoids. In this instance each of the dry extract residues was injected into the HPLC system and elution was monitored by an on-line radioactive HPLC detector. The values shown correspond to the detector response (in counts/s) for each prostanoid; there was no significant difference between the manual and automated ASPEC extraction systems. The relative standard deviations (R.S.D.) are at least equivalent or better than those obtained by manual processing.

A possible limitation of automated batch sequential processing of 36 samples could be the degradation of the analytes to be extracted and analysed. In the ASPEC system this is carried out in batch mode using the same steps as in the manual mode, namely individual sample loading into each extraction column, column washing and finally elution of the retained prostanoids. This implies that in the approximately 2 h that the whole process takes, the samples could undergo degradation and autoxidation, thus affecting the determination. In contrast, in the manual processing, the analytes once loaded do not have to wait for other samples to be loaded and washed and thus spend the minimum possible time

TABLE II

COMPARISON BETWEEN AUTOMATED (ASPEC) AND MANUAL EXTRACTION RECOVERIES OF PROSTANOIDS (6-KETO-PFG$_{1\alpha}$, TXB$_2$, PGF$_{2\alpha}$, PGE$_2$ y PGD$_2$) IN URINE

Results expressed as mean ± standard deviation (intra-assay R.S.D.); n = 10.

	Recovery (%)		
	Manual extraction, 400 mg absorbent	Manual extraction, 100 mg absorbent	ASPEC extraction, 100 mg absorbent
6-keto-PGF$_{1\alpha}$	93.0 ± 2.0 (2.1%)	89.6 ± 1.1 (1.2%)	92.6 ± 1.2 (1.2%)
TXB$_2$	95.7 ± 4.1 (4.2%)	85.9 ± 5.7 (6.6%)	92.3 ± 2.0 (2.1)
PGE$_2$	93.7 ± 3.2 (3.4%)	95.1 ± 1.8 (1.9%)	91.6 ± 1.1 (1.2%)
PGF$_{2\alpha}$	94.0 ± 3.0 (4.3%)	93.6 ± 2.1 (2.1%)	90.2 ± 2.9 (3.2%)

TABLE III

COMPARISON OF ABSOLUTE RESPONSES OF THE RADIOACTIVITY HPLC DETECTOR FOR EACH OF THE FIVE TRITIUM-LABELLED PROSTANOIDS ADDED TO HUMAN URINE SAMPLES

Values under the manual or ASPEC headings represent the results obtained after manual processing of the urine samples *versus* ASPEC extraction of aliquots of the same samples.

Compound	Response (counts/s)	
	Manual	ASPEC
$PGF_{2\alpha}$	1467	1489
	1139	1143
	1548	1588
	1537	1545
	1505	1523
	946	855
	2056	1539
Mean	1457	1383
S.D.	350	277
R.S.D. (%)	24	20
PGE_2	1386	1440
	1296	1198
	1523	1380
	1451	1442
	1581	1530
	1071	924
	1922	1584
Mean	1461	1357
S.D.	263	222
R.S.D. (%)	18	16.3
6-Keto-$PGF_{1\alpha}$	868	787
	681	675
	816	897
	814	890
	1213	1200
	523	456
	1068	1175
Mean	855	868
S.D.	230	264
R.S.D. (%)	26.9	30.4
TXB_2	702	553
	533	470
	496	607
	565	632
	596	594
	395	396
	863	667
Mean	593	560
S.D.	151	96
R.S.D. (%)	25.4	17.1

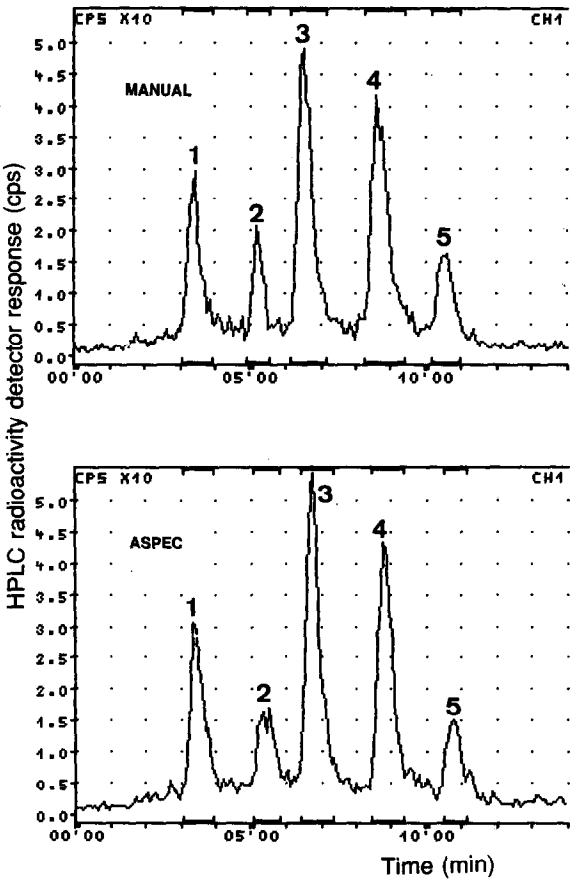

Fig. 1. Representative radiochromatograms of tritiated prostanoid standards in urine samples previously extracted by the manual (top) or ASPEC procedure (bottom).The HPLC mobile phase was a mixture of 40 m*M* formic acid (pH 3.15) with triethylamine–acetonitrile (65:35, v/v) at a flow-rate of 1 ml/min. Peaks: 1 = 6-keto-$PGF_{1\alpha}$; 2 = TXB_2; 3 = $PGF_{2\alpha}$; 4 = PGE_2; and peak 5 = PGD_2.

TABLE IV

URINARY CONCENTRATIONS (pg/ml) OF TXB_2 AND PGE_2 IN WORKERS EXPOSED TO DICHLOROETHANE (n = 12) COMPARED WITH UNEXPOSED CONTROLS (n = 12)

Urine samples were extracted with the ASPEC system and were analysed by HPLC–RIA. Results are expressed as mean ± S.E.M.; Student's t-test was used; ns = not significant.

	Worker exposed to dichloroethane	Control subject
TXB_2	148.1 ± 17.6	93.7 ± 13.2 (ns)
PGE_2	74.9 ± 49.7	94.9 ± 53.3 (ns)

in the extraction column before finally being eluted into the methyl formate fraction. However, the almost identical radiochromatograms obtained for the manual and automated sample extraction modes (Fig. 1) show that the automatic system does not affect the stability of these analytes.

The automated ASPEC technique has been used in conjunction with an HPLC–RIA procedure [6] to determine TXB_2 and PGE_2 in urine from workers exposed to dichloroethane.

Prostanoids could be used as an early marker of environmentally induced nephrotoxicity. Although a detailed account of this work showing that prostanoid urinary excretion is altered in workers exposed to heavy metal pollution will be published elsewhere, for dichloroethane no significant differences were found relative to healthy control subjects (Table IV).

In conclusion, the automated extraction of prostanoids from urine samples has been shown to be feasible using a commercially available ASPEC system. Compared with manual extraction methods reported previously the method uses less organic solvents and has a shorter processing time while maintaining equivalent or lower inter- and intra-assay variation coefficients.

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