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Note

Extraction of urinary leukotriene E_4 by the combined use of octadecyl reversed-phase and NH_2 normal-phase extraction columns

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Leukotrienes (LTs) are a family of biologically active metabolites derived from arachidonic acid and related long-chain polyunsaturated fatty acids through initial oxygenation of lipoxygenase [1]. Among them, sulphidopeptide leukotrienes LTC_4, LTD_4 and LTE_4 have been implicated in a wide range of diseases, such as allergy, trauma, respiratory infection, myocardial infarction and persistent pulmonary hypertension of the newborn [2–6]. These LTs are formed after immunological as well as non-immunological stimulation, and can alter smooth muscle tone and increase vascular permeability [1]. A proportion of the peptide leukotriene metabolites in humans are excreted as LTE_4 into the urine within several hours after either intravenous injection of radiolabelled LTC_4 or inhalation of non-radiolabelled LTD_4 [7–9]. Accordingly, clinical studies have recently been performed through the analysis of urinary LTE_4, a stable end-product of LTC_4 and LTD_4, to avoid any LT-like immunoreactive impurity in plasma or artificial formation of LTs at blood sampling [5,10,11]. In these studies, attempts were made to exclude the non-specific LTE_4-like immunoreactivity present in urine by using octadecyl reversed-phase extraction cartridges in high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA) with radiolabelled LTE_4 as an internal standard.

Previous experience with the measurement of prostaglandins and thromboxanes in biological samples, particularly in urine [12], has provided some important insights. As a case in point, the specificity of the assay method for prostaglandins and thromboxanes has usually been confirmed by gas chromatography–mass spectrometry (GC–MS). However, high-performance liquid chromatography–mass spectrometry (HPLC–MS), which is a method corresponding to GC–MS, apparently lacks the sensitivity to check accurately the small amounts of LTE_4 present in urine measured by RIA [13]. Therefore, we adopted a further purification step in addition to the reported assay method by

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octadecyl reversed-phase extraction cartridges coupled with HPLC, and RIA with radiolabelled LTE_4 as an internal standard. We studied the specificity of the reported assay method for urinary LTE_4 through differences in the behaviour of non-specific immunoreactivity and urinary LTE_4 concentrations between the reported assay method and those with an additional purification step.

EXPERIMENTAL

Chemicals

The following drugs were used: synthetic LTB_4, LTC_4, LTD_4 and LTE_4 (Cayman, Ann Arbor, MI, U.S.A.). Octadecyl reversed-phase extraction columns (Sep-Pak C_{18} cartridges), silica normal-phase extraction columns (Sep-Pak silica cartridges) and NH_2 normal-phase extraction columns (Sep-Pak light NH_2 cartridges) were purchased from Waters Assoc. (Milford, MA, U.S.A.). Solvents were of HPLC grade (Nacalai Tesque, Kyoto, Japan). All other chemicals used were of HPLC grade.

Collection of urine and extraction of LTE_4

Urine was sampled from nine (four males and five females) healthy and drug-free adult volunteers without a history of allergic diseases. Samples were taken early in the morning before breakfast and stored at $-60°C$ until later use. Immediately before assay, urine samples were thawed, spiked with 6000 dpm ^3H-labelled LTE_4 (Amersham International, 56 Ci/mmol) per 60 ml of urine as an internal standard and centrifuged at 45 000 *g* for 10 min at 4°C. As a first step, the supernatant was extracted without acidification with a Sep-Pak C_{18} cartridge, which was initially conditioned with 10 ml of methanol, 5 ml of water and then 5 ml of 0.1% EDTA solution [14]. The cartridge was washed with 10 ml of water followed by 10 ml of 50% methanol solution, and the adsorbed lipid was eluted with 3 ml of methanol. The 3-ml methanol fraction was diluted with 1 volume of methanol, and then divided into two equal fractions. One part of the diluted methanol fraction was applied to a Sep-Pak light NH_2 cartridge for an additional extraction step. The cartridge was washed with 6 ml of methanol, and the urinary LTE_4 fraction was finally eluted with 10 ml of 0.5% acetic acid–methanol solution.

In a preliminary experiment to screen appropriate extraction columns for the additional step after extraction with a Sep-Pak C_{18} cartridge, the recovery of synthetic LTE_4 in methanol solution was also examined using a silica extraction column (Sep-Pak silica cartridge). However, the extraction of LTE_4 with a Sep-Pak silica cartridge was hindered by several artifactual peaks absorbing at 280 nm and eluting near to or with the synthetic LTs. This phenomenon, which was noticed by Metz *et al.* [15], could reportedly be prevented by tedious pre-washing of the Sep-Pak silica cartridges. Additionally, the extraction by Sep-Pak silica cartridges resulted in poorer radioactive recovery than that by Sep-Pak light NH_2

cartridges, probably because of stronger binding of the former cartridges. Conversely, no apparent artifactual peaks absorbing at 280 nm were found in the case of extraction with Sep-Pak light NH_2 cartridges. Hence, the latter were used for additional extraction of urinary LTE_4.

Both the 3-ml fraction from the Sep-Pak C_{18} cartridge and the 6-ml 0.5% acetic acid–methanol fraction from the Sep-Pak light NH_2 cartridge were carefully evaporated to *ca.* 100 μl under a stream of nitrogen gas. Complete drying resulted in poor recovery, which might be explained by the well known phenomenon that some lipids are readily adsorbed on glass walls.

Chromatography and RIA

The HPLC equipment consisted of a Model M6000 pump, a U6K injector and a 990J computerized photodiode array spectrophotometer (all from Waters Assoc.). The mobile phase for HPLC was methanol–distilled water–acetic acid (65:35:0.10, v/v), titrated to pH 4.9 with ammonium hydroxide. The aqueous phase contained 0.1% EDTA, which improved the recovery of sulphidopeptide leukotrienes by preventing binding of cations to the column [14].

The separations were carried out using a Cosmosil $5C_{18}$ packed column (Nacalai Tesque, 150 mm × 4.6 mm I.D., particle size 5 μm) and a constant flow-rate of 1.0 ml/min. The column oven was maintained at 38°C during chromatography. A $10C_{18}$ packed guard-column was used at all times. UV adsorbance was continuously monitored in the range 240–320 nm, and the mixtures of synthetic LTB_4, LTC_4, LTD_4 and LTE_4 were chromatographed separately to allow identification of the tritiated products. Column effluent was collected in 2-ml fractions after the extracted urine samples were injected. Each fraction was evaporated to near dryness under vacuum, resuspended in 300 μl of immunoassay buffer, then divided into two parts: one third was used to determine the radioactive recovery with a liquid scintillation spectrometer (Model LSC-703, Aloka, Japan) and the rest was assayed in duplicate for LTE_4 immunoreactivity. RIA was carried out, essentially as described in the manufacturer's instructions, with the $LTC_4/D_4/E_4[^3H]$ assay kit (Amersham, U.K.). Amersham International report that their antiserum had the following cross-reactivities: LTC_4, 100%; LTD_4, 49%; LTE_4, 40%. The radioactive LTE_4 added into urine samples as internal standard gave little, if any, interference with RIA. The concentrations of urinary LTE_4 were expressed as pg/ml of urine, after correction by radioactive recovery from initial addition into urine to post-HPLC column elution.

A paired Student's *t*-test was used to evaluate the significance in the results for urinary LTE_4 concentrations.

RESULTS

Chromatography

Fig. 1A shows a representative immunochromatogram of urine after a single

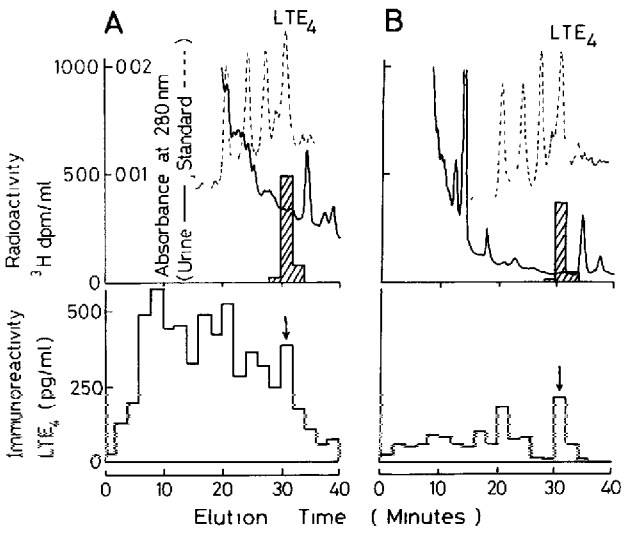

Fig. 1 Representative chromatograms of urine samples from healthy volunteers, showing UV absorbance at 280 nm, LTE_4 immunoreactivity and radioactivity (from ^3H-labelled internal standard). (A) Chromatogram of a urine sample extracted with a Sep-Pak C_{18} cartridge and resolved by HPLC (B) Chromatogram of a urine sample extracted with Sep-Pak C_{18} and Sep-Pak NH_2 and resolved by HPLC. Dashed lines show HPLC profiles of synthetic LTB_4, LTC_4, LTD_4 and LTE_4

extraction with a Sep-Pak C_{18} cartridge, showing LTE_4, together with the radioactive chromatogram from [^{3}H]LTE_4 and UV absorbance at 280 nm of the urine extract. For reference, a typical reversed-phase HPLC separation of synthetic mixtures (LTB_4, LTC_4, LTD_4 and LTE_4) detected at 280 nm is also shown as a dashed line. The largest radiochromatogram peak coincided with the peak of synthetic LTE_4 with a retention time of *ca.* 31 min, and significant immunoreactivity was found in the corresponding fraction. However, overwhelming amounts of non-specific immunoreactivity were also identified in other fractions devoid of synthetic LTE_4, as defined by the absence of the internal standard, [^{3}H]LTE_4.

The non-specific immunoreactivity in the urine extract was substantially decreased by the combined use of Sep-Pak C_{18} and Sep-Pak light NH_2 cartridges, as shown in Fig. 1B. A large immunoreactive peak was found, with a retention time of 26 min, at fractions corresponding to the radioactive peak from [^{3}H]LTE_4. The retention time of this peak also seemed to coincide with that of the synthetic LTE_4 peak, the UV absorbance (280 nm) of which is shown as a dashed line for reference.

However, the co-elution of the immunoreactive peak with synthetic LTE_4 (as measured by UV) was proven to be incorrect in a separate experiment. Several urine samples were extracted by the combined use of Sep-Pak C_{18} and Sep-Pak light NH_2 cartridges with and without initial addition of synthetic LTE_4 to urines, and HPLC fractions were collected at 0.5-ml intervals instead of 2-ml intervals. The retention time of the immunoreactive peak, both with and without

initial addition of synthetic LTE_4, also coincided with that of the radioactive peak, but it was *ca.* 0.5 min later than that of synthetic LTE_4 (as measured by UV), probably reflecting a time-lag between UV detection and fraction collection (data not shown).

Concentrations of urinary LTE_4

The recoveries of [^{3}H]LTE_4 internal standard from 60 ml of urine that were extracted only with a Sep-Pak C_{18} cartridge or by the combined use of Sep-Pak C_{18} and Sep-Pak light NH_2 cartridges were *ca.* 40% and *ca.* 50%, respectively, after resolution by HPLC. Table I, shows that the concentrations of urinary LTE_4 extracted only with Sep-Pak C_{18} cartridges were significantly higher than those obtained by combined extraction with both cartridges (236 ± 150 *versus* 72 ± 44 pg/ml; $p < 0.01$).

DISCUSSION

In the analysis of urinary leukotrienes, Taylor *et al.* [5] and others [10,16] acknowledged the presence of LTE_4 only when a concomitant rise and fall in immunoreactivity and radioactivity was noted across the HPLC profile, thereby emphasizing the structural assignment of LTE_4. First, our criteria for immunoreactive LTE_4 in urine was essentially similar to theirs, *i.e.* using a similar assay

TABLE I

EFFECT OF DIFFERENT EXTRACTION METHODS ON THE LEVELS OF URINARY LTE_4 RESOLVED BY HPLC AND ASSAYED BY RIA

Volunteer	Concentration of LTE_4 (pg/ml of urine)		A/B ratio[a]
	Sep-Pak C_{18} (A)	Sep-Pak NH_2 (B)	
Males			
1	227	89	2.6
2	341	78	4 4
3	183	38	4 8
4	265	48	5 5
Females			
1	98	29	3 5
2	248	51	4.9
3	61	32	1 9
4	143	134	1 1
5	562	146	3.9

[a] Comparison was made between the values obtained after extraction with (A) only Sep-Pak C_{18} cartridges and (B) combined use of Sep-Pak C_{18} and Sep-Pak light NH_2 cartridges. $p < 0.01$

method with Sep-Pak C_{18} extraction, reversed-phase HPLC with [^{3}H]LTE_4 as internal standard, followed by RIA, except that their extraction was automatic whereas ours was manual. Later, however, with an additional extraction step with Sep-Pak light NH_2, we obtained a substantial improvement in the immunochromatogram and UV chromatogram of urinary LTE_4, as evidenced by the disappearance of the non-specific LTE_4-like immunoreactivity and UV absorbance, eluting at times other than the radioactive or non-radioactive internal standard. Thus our results show that co-elution of immunoreactive LTE_4 with the [^{3}H]LTE_4 internal standard is indeed required for assuring specificity of the assay method, though not necessarily satisfactory. Often, extraction with Sep-Pak C_{18} alone yielded higher values than those obtained by the combined use of Sep-Pak C_{18} and Sep-Pak light NH_2 cartridges. This would afford good quantitative evidence against the pitfall in the analysis described above. Of course, the specficity of the assay method cannot be defined in absolute terms, since it is hard to determine all sources of interference.

Another important factor responsible for the specificity of the urinary LTE_4 assay seems to be the inherent specificity of antiserum against LTE_4 available for RIA. In this study, we used a commercially available one, which is more specific for LTC_4 and LTD_4 than for LTE_4. With more specific antiserum against LTE_4, such as monoclonal antibody against LTE_4, we might have obtained less difference in urinary LTE_4 values between the two kinds of extraction method.

In conclusion, our improved extraction method for urinary LTE_4 illustrates the importance of specificity in the assay method. This method would also be applicable to quantitative MS analysis of urinary LTE_4 by HPLC–MS, since samples for MS analysis usually require more purification steps than those for RIA.

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