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Improvement of chemical analysis of antibiotics

XX☆. Basic study on high-performance liquid chromatographic determination of four polyether antibiotics pre-derivatized with 1-bromoacetylpyrene

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ABSTRACT

A high-performance liquid chromatographic method for the determination of the polyether antibiotics (PEs), salinomycin (SL), monensin (MN), lasalocid (LA) and narasin (NA), based on a precolumn reaction system using 1-bromoacetylpyrene (1-BAP) as a fluorescence reagent, was established. Six standards of 1-pyrenacyl esters (PEs-PYs) including two of 1-pyrenacyl esters of internal standards, 18,19-dihydrosalinomycin (DSL) and 18,19-dihydro-20-ketosalinomycin (DKSL), were separated within 30 min on a Develosil $5C_{18}$ (5 μm) column (250 × 4.6 mm I.D.) with methanol–water (97:3) as the mobile phase at a flow-rate of 1.0 ml/min and were detected at λ_{ex} 360 nm, λ_{em} 420 nm. This system was also effective for the separation between unused 1-BAP and PEs-PYs and the simultaneous determination of SL, MN, LA and NA was achieved at concentrations from 0.2 to 100 μg/ml. At concentrations of less than 10 μg/ml a silica gel cartridge was effective in eliminating the excess of unused reagents. Four calibration graphs with the internal standard method were linear between 20 and 100 μg/ml, 2 and 10 μg/ml and 0.2 and 1.0 μg/ml. The method is applicable to feed and residue analyses.

INTRODUCTION

Polyether antibiotics (PEs) (Fig. 1) are produced by *Streptomyces* and structurally characterized as the sodium salt of a carboxylic acid ionophore and a number of cyclic ether moieties. Among them salinomycin (SL), monensin (MN), lasalocid (LA) and narasin (NA) have microbial activities against Gram-positive bacteria and fungi, and have been used all over the world as feed additives for poultry to prevent coccidiosis [1].

Many analytical method for PEs have been reported since the early 1970s. Most of them mainly involved the detection of these compounds, because most PEs have no chromophore. Several groups have performed HPLC determinations of SL, MN and NA using refractive index detection or UV detection at 214 nm [2–4]. Vanillin and *p*-dimethylaminobenzaldehyde have often been used in the conventional spectrophotometry of SL, MN and NA [5–11]. Under anhydrous acidic conditions, SL, MN,

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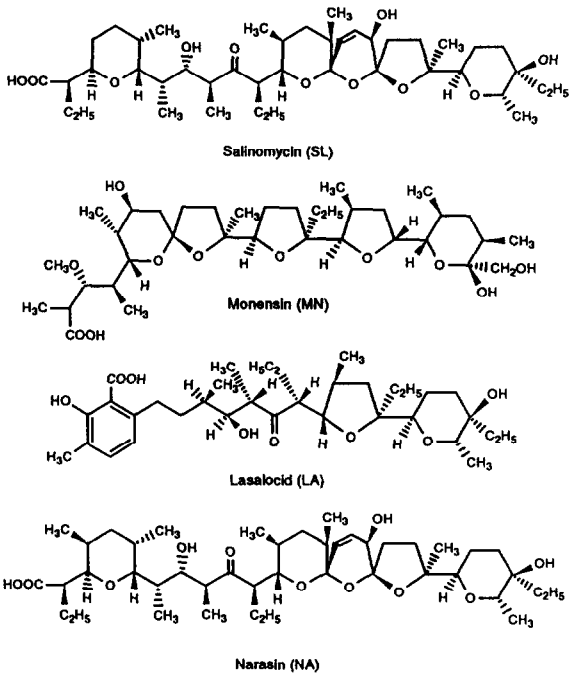

Fig. 1. Structures of polyether antibiotics (PEs).

and NA react with these reagents to form products that show strong absorption maxima in the visible region. Since Goras and Lacourse [12] reported an HPLC determination of SL using postcolumn derivatization with vanillin, many workers have used this technique for the simultaneous HPLC determination of SL, MN and NA [13–18]. LA shows significant fluorescence in various organic solvents, which has been applied in spectrofluorimetric [19–22] and HPLC with fluorescence detection methods [23–27]. However, LA is less reactive to the reagent than SL, MN and NA [28], indicating that it is difficult to determine PEs including LA simultaneously using postcolumn derivatization. Additionally, several groups tried to establish HPLC for MN with fluorescence detection using derivatization with 9-anthryldiazomethane (ADAM) [29–35], which reacts rapidly with the free carboxylic acids of PEs but it does not react with the carboxylates. Although conversion into the free carboxylic acid is possible by treatment with acidic buffer, MN is unstable during the process.

Two papers on the simultaneous determination of SL, MN, LA and NA have been published. Johannsen [16] established a sequential detection system consisting of UV detection (320 nm) for LA and postcolumn derivatization with detection at 600 nm. However, the system requires three pumps and two detectors and no actual analysis was performed using this method. Martinez and Shimoda [32] tried a multi-residue determination of these compounds, in which LA was directly esterified with ADAM, but SL, MN and NA were first acetylated followed by esterification with ADAM. It is difficult to understand why acetylation is necessary for SL, MN, and NA. Probably the method would not be directly applicable to an actual mixture of these PEs. As stated above, no practical method for the simultaneous HPLC determination of PEs including SL, MN, LA and NA has been established so far.

In a previous study, we established a high-performance thin-layer chromatographic (HPTLC) method for the simultaneous fluorodensitometric determination of SL, MN and LA based on fluorescence labelling with 1-bromoacetylpyrene (1-BAP) using 18,19-dihydrosalinomycin (DSL) and 18,19-dihydro-20-ketosalinomycin (DKSL) as internal standards (Fig. 2) [36]. Although the HPTLC method was simple and reliable, it is not always sensitive enough to allow the detection of small amounts of these compounds with good precision. Further, we have not applied the method to NA, because it has not been approved for use as a feed additive in poultry in Japan.

On the basis of the above considerations, we tried to establish an HPLC method for the determination of four PEs with fluorescence

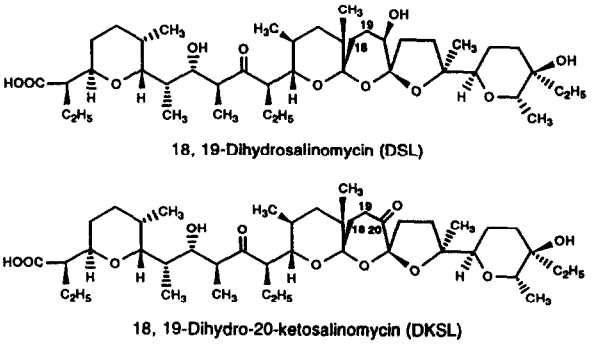

Fig. 2. Structures of internal standards (IS).

detection which is applicable to feed and residual analyses. This paper describes the optimum conditions and techniques utilized for the identification and determination of the four PEs by HPLC.

EXPERIMENTAL

Chemicals

SL and NA were supplied by Pfizer Pharmaceuticals (Tokyo, Japan), and Lilly Research Laboratories (Indianapolis, IN, USA), respectively. MN and LA were purchased from Hexyst-Japan (Tokyo, Japan) and Sigma (Tokyo, Japan), respectively. DSL and DKSL were prepared according to the procedure described in a previous paper [36]. 1-BAP [37] was prepared according to the procedure of Kawahara *et al.* [37] (1-BAP is now available from Wako, Osaka, Japan). Kryptofix 222 (K-222; 4,7,13,16,21,24-hexaoxy-1,10,diazobicyclo[8,8,8]hexacosane) was purchased from Merck (Darmstadt, Germany). A Mega Bond Elut silica gel cartridge was purchased from Varian (Harbor City, CA, USA). All other chemicals were of analytical-reagent grade.

Apparatus

An HPLC system equipped with a constant-flow pump (Trirotor; JASCO, Tokyo, Japan) was used together with a spectrofluorimetric detector (FP-210; JASCO) operated at $\lambda_{ex} = 360$ nm and $\lambda_{em} = 420$ nm. The separation was performed on Develosil $5C_{18}$ (5 μm) (Nomura Chemical, Seto, Japan) packed in a stainless-steel column (250 × 4.6 mm I.D.) using methanol–water (97:3) as the mobile phase.

Preparation of polyether pyrenacyl esters (PEs-PYs)

1-BAP (19.4 mg, 0.060 mmol) and K-222 (12.2 mg, 0.032 mmol) were added to a solution of NA (10.3 mg, 0.013 mmol) in acetonitrile (5 ml). The solution was stirred for 150 min and then concentrated under reduced pressure. The residue was chromatographed on a silica gel column (150 × 10 mm I.D.) (Merck) with benzene–acetone (9:1) to give narasin 1-pyrenacyl ester (NA-PY, 15.7 mg) as colourless needless; fast atom bombardment mass spectrometry: *m/z* 1029 $(M + Na)^+$, 1007 $(M + H)^+$ and 989 $(M + H - H_2O)^+$. The preparation of other polyether pyrenacyl esters (PEs-PYs) has been described in previous papers [36,38].

Preparation of standard and internal standard solutions

Amounts of 10 mg each of standards (SL, MN, LA and NA) and internal standards (DSL and DKSL) were accurately weighed into two 50-ml volumetric flasks and diluted to volume with methanol (200 μg/ml). In the simultaneous determination of concentrations of less than 10 μg/ml, subsequent dilutions were made with methanol to give the desired concentrations.

Preparation of 1-BAP solution

An amount of 132 mg of 1-BAP was dissolved in 25 ml of acetonitrile.

Preparation of K-222 solution

An amount of 32 mg of K-222 was dissolved in 25 ml of acetonitrile.

Clean-up procedure for elimination of reagent

After cooling a round-bottomed flask containing the reaction mixture to room temperature, the mixture was evaporated to dryness and the residue was dissolved in 5 ml of benzene–chloroform (1:1) followed by rinsing twice with 5 ml of the same solvent. After filtration of the solution, the filtrate was applied to a Mega Bond Elut silica gel cartridge activated with benzene. PEs-PYs were eluted twice with 5 ml of benzene–acetone (7:3). After evaporation of the eluate to dryness, the residue was dissolved in 10 ml of acetonitrile and the determination was carried out by HPLC.

Simultaneous determination

Volumes of 1, 2, 3, 4 and 5 ml of the standard solution (200 μg/ml) were pipetted into 20-ml round-bottomed flasks and 3 ml of internal standard solution (200 μg/ml) were added by pipette. After evaporation of the solutions to dryness, 5 ml of 1-BAP solution and 5 ml of K-222 solution were added to each flask. In this step, concentrations of standards were 20, 40,

60, 80 and 100 μg/ml, respectively, and those of internal standards (DSL and DKSL) were 60 μg/ml. The flasks were allowed to stand in an oil-bath at 50°C for 90 min. After cooling the flasks to room temperature, the reaction mixtures were subjected to HPLC.

In the simultaneous determination of concentrations ranging from 2 to 10 μg/ml and from 0.2 to 1.0 μg/ml, the concentrations of standard solution and internal standard solution were 20 and 2 μg/ml, respectively. After the derivatization, the cooled derivatizing solution was evaporated to dryness and was subjected to the clean-up procedure described above.

RESULTS AND DISCUSSION

Separation

In order to optimize the separation conditions, authentic pyrenacyl esters (PEs-PYs) of six PEs were first prepared according to our method described previously [36,38] and their structures were confirmed by mass spectrometry. Second, optimum separation conditions for the six pyrenacyl esters (SL-PY, MN-PY, LA-PY, NA-PY, DSL-PY and DKSL-PY) were sought and the separation between the derivatization reagent (1-BAP) and PEs-PYs was also investigated.

We attempted to apply our previously reported solvent system [dichloromethane–ethyl acetate–acetone–acetonitrile (15:2:1:55)] for reversed-phase HPTLC (RP-HPTLC) [36] to HPLC analysis using a C_{18} column in this work. However, sufficient resolution between 1-BAP and PEs-PYs could not be obtained, because the capacity factors were too small. The different behaviour can be explained by the assumption that the separation by RP-HPTLC is based not only on a partitioning effect but also adsorption to residual silanol groups on surface of the stationary phase. Hoshino *et al.* [30] reported that a mobile phase containing methanol gave better results than one containing acetonitrile when MN derivatized with ADAM was separated by HPLC on a C_{18} column. Therefore, various mobile phases containing methanol were tried and a satisfactory separation of the six PEs-PYs was obtained using methanol–water (97:3). There are four minor peaks in the chromatogram (Fig. 3), peaks 1, 2 and 3 between DKSL-PY and SL-PY and peak 4 behind of NA-PY. Peak 1 was identified as the pyrenacyl ester of 20-ketosalinomycin (KSL-PY), which is a minor contaminant of DKSL. The other peaks have not been identified, but peaks 2, 3 and 4 are contaminants derived from DKSL, SL and NA, respectively. However, as they do not interfere in the determination of PEs-PYs, the separation between the derivatizing reagents and the PEs-PYs was examined using this mobile phase.

After derivatization of the six PEs at concentrations of 100 μg/ml under the conditions described under Experimental, the reaction mixture was directly injected into the HPLC system. As shown in Fig. 4, new minor peaks 5 and 6 appeared and peak 1 (KSL-PY) and a minor peak derived from 1-BAP overlapped (indicated by an arrow). However, a good separation of the PEs-PYs was obtained apart from the peaks

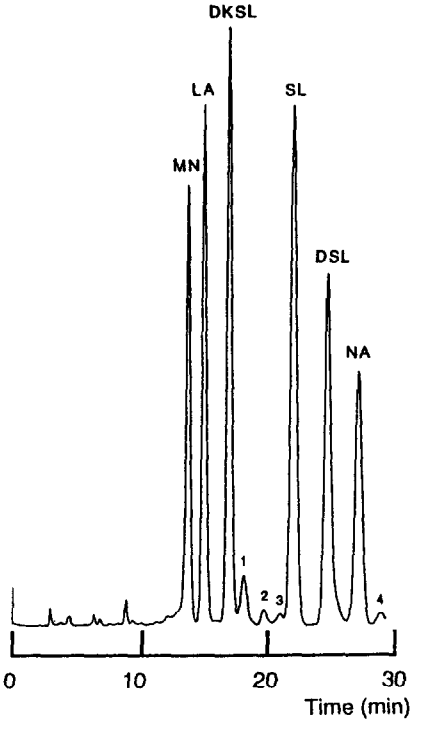

Fig. 3. HPLC separation of pyrenacyl esters of polyether antibiotics (PEs-PYs). Column, Develosil $5C_{18}$ (250 × 4.6 mm I.D.); mobile phase, methanol–water (97:3); flow-rate, 1 ml/min; detection, λ_{ex} = 360 nm and λ_{em} = 420 nm.

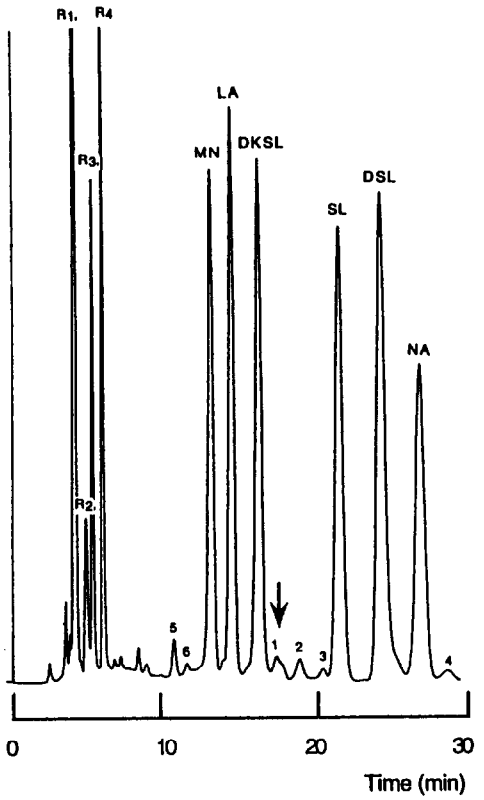

Fig. 4. Typical chromatogram of simultaneous determination (100 μg/ml).

around 5 min, which are derived from the reagent, and these minor peaks including peaks 1–6 did not interfere in the determination of PEs. These results indicate that a combination of a C_{18} column and methanol–water (97:3) as the mobile phase is suitable for the present purpose.

Derivatization

In order to determine the reaction yield of the derivatization, calibration graphs for the six PEs-PYs were constructed for concentration from 20 to 100 μg/ml, from 2 to 10 μg/ml, from 0.2 to 1.0 μg/ml and from 0.02 to 0.1 μg/ml. With the exception of the most sensitive case, the three cases showed correlation coefficients of >0.999, so the reaction yield could be calculated from these calibration graphs.

In a previous study [36], the following derivatization conditions were used: molar excess of reagent (1-BAP), 50-fold; molar excess of catalyst (K-222), 10-fold; reaction temperature, 50°C; reaction time, 90 min. We applied these conditions to the simultaneous derivatization of the six PEs at concentrations of 100, 10 and 1.0 μg/ml and calculated the reaction yields (Table I). At a concentration of 100 μg/ml, each reaction showed a yield of >90%. However, in the reaction of LA the yield decreased to <50% at a concentration of 10 μg/ml, and those of MN and LA were only 6% and 2%, respectively, at a concentration of 1 μg/ml. When the derivatization is applied to feed and residue analyses, it is necessary to have a high reaction yield even at low concentrations. We used more than 10–100-fold excesses of reagent and catalyst (100 μg/ml) to obtain higher reaction yields at analyte concentrations of 10 and 1 μg/ml, however, no peaks for the six kinds of PEs-PYs were found because of the peaks of unused reagents present on the chromatogram.

In order to eliminate the excess of reagent, the difference in the polarities of PEs-PYs and 1-

TABLE I

DERIVATIZATION CONDITIONS AND REACTION YIELD OF PEs WITH 1-BAP

Amount of PEs (μg/ml)	Molar excess (-fold)		Yield (%)[a]					
	Reagent	Catalyst	SL	MN	LA	NA	DSL	DKSL
100	50	10	96	100	94	102	101	90
10	50	10	96	85	50	105	100	91
1	50	10	80	6	2	76	72	59
10	500	100	93	98	98	104	99	94
1	5000	1000	84	85	68	85	82	80

[a] Reaction yield was calculated from calibration graphs for authentic pyrenacyl esters.

BAP was efficiently employed for a clean-up using a silica gel cartridge. When benzene was used as the mobile phase for silica gel TLC, the spots of PEs-PYs remained at the origin. Whereas 1-BAP showed an R_F value of 0.70. This chromatographic behaviour was applied with the above objective, but the amount of unused reagent was so large that the evaporated reaction mixture showed poor solubility in benzene. Although the reaction mixture was soluble in chloroform, LA was eluted first with this solvent. Therefore, chloroform–benzene (1:1) was chosen as the solvent based on the solubility of the reaction mixture and polarities of 1-BAP and PEs-PYs. Finally, the following procedure was established: after the derivatization, the rection mixture was evaporated to dryness and the residue was dissolved in 5 ml of chloroform–benzene (1:1). The solution containing PEs-PYs and 1-BAP was applied to the silica gel cartridge, followed by rinsing twice with 5 ml of the same solvent, and PEs-PYs were eluted twice with 10 ml of benzene–acetone (7:3).

To investigate the suitability of this clean-up procedure, the reaction yield of PEs was calculated at concentrations of 10 and 1 μg/ml. The reaction yield obtained is shown at bottom of Table I. Although a peak derived from 1-BAP appeared close to that of SL-PY (indicated by an arrow, Fig. 5) at the concentration of 1 μg/ml, it did not interfere in the separation of PEs-PYs. The reaction yield of LA at the concentration of 1 μg/ml was 68%, but those of the others were >80%, indicating that the derivatization system coupled with the described clean-up procedure is effective for the simultaneous determination of SL, MN, LA and NA.

Simultaneous determination of SL, MN, LA and NA by HPLC

We achieved the simultaneous determination of SL, MN, LA, and NA at concentrations ranging from 20 to 100 μg/ml, from 2 to 10 μg/ml and from 0.2 to 1.0 μg/ml under the conditions mentioned above using DSL and DKSL as internal standards. At concentrations between 20 and 100 μg/ml the reaction mixture was introduced directly into the HPLC system, and at concentrations between 2 and 10 μg/ml and between 0.2 and 1.0 μg/ml the samples were subjected to HPLC after treatment with the silica gel cartridge as described above. The data in Table II indicate a good correlation for the four PEs with the two internal standards. In a previous study [36], the internal standard DSL was effective for SL and MN and DKSL for LA. However, both internal standards were effective for the four PEs in the present study, indicating that these two internal standards can be used complementarily depending on the nature of the sample where many interfering substances may exist.

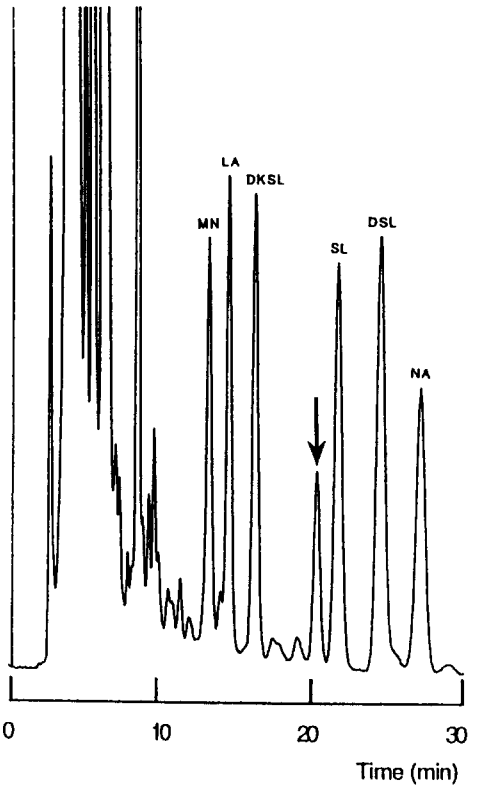

Fig. 5. Typical chromatogram of simultaneous determination (1 μg/ml).

CONCLUSIONS

A technique using HPLC with fluorescence detection and prederivatization with 1-BAP has been established for the determination of four

TABLE II

CHARACTERISTICS OF CALIBRATION GRAPHS OF POLYETHER ANTIBIOTICS (PEs) USING THE INTERNAL STANDARDS 18,19-DIHYDROSALINOMYCIN (DSL) AND 18,19-DIHYDRO-20-KETOSALINOMYCIN (DKSL)

Sample	Internal standard	20–100 μg/ml		2–10 μg/ml		0.2–1.0 μg/ml	
		Regression equation[a]	Correlation coefficient	Regression equation[a]	Correlation coefficient	Regression equation[a]	Correlation coefficient
Salinomycin (SL)	DSL	$y = 0.015x + 0.810$	0.9995	$y = 0.171x + 0.006$	1.0000	$y = 1.682x + 0.063$	0.9996
	DKSL	$y = 0.016x + 0.081$	0.9998	$y = 0.147x - 0.000$	0.9999	$y = 1.476x + 0.028$	0.9995
Monensin (MN)	DSL	$y = 0.013x + 0.015$	0.9999	$y = 0.152x - 0.091$	0.9996	$y = 1.370x + 0.030$	0.9993
	DKSL	$y = 0.015x + 0.005$	0.9998	$y = 0.131x - 0.018$	0.9997	$y = 1.202x + 0.004$	0.9993
Lasalocid (LA)	DSL	$y = 0.017x + 0.028$	0.9996	$y = 0.174x + 0.002$	0.9996	$y = 1.698x - 0.011$	0.9998
	DKSL	$y = 0.018x + 0.024$	0.9998	$y = 0.015x - 0.003$	0.9995	$y = 1.489x - 0.035$	0.9989
Narasin (NA)	DSL	$y = 0.011x + 0.032$	0.9998	$y = 0.125x + 0.018$	1.0000	$y = 1.253x + 0.044$	0.9998
	DKSL	$y = 0.012x + 0.029$	1.0000	$y = 0.108x + 0.012$	1.0000	$y = 1.099x + 0.018$	0.9994

[a] y = Ratios of peak heights of sample and I.S.; $x = \mu g/ml$.

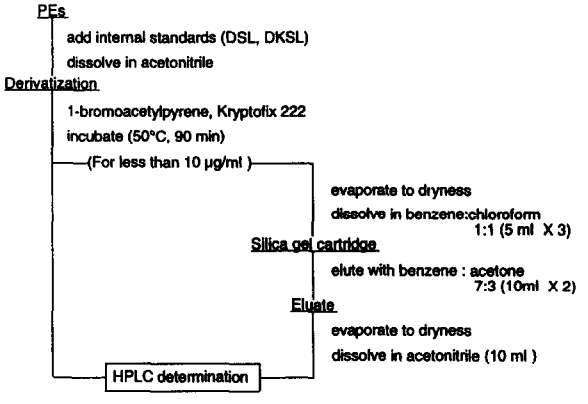

Fig. 6. Analytical procedure for PEs prederivatized with 1-BAP.

PEs. The analytical procedure is summarized in Fig. 6. We consider that this derivatization system is more reliable than that with ADAM. The established method would be a more reliable method for the determination of SL, MN, LA and NA in feed and residue analyses. We have performed feed analyses for SL, MN, and LA using this separation system and the results will be reported elsewhere [39].

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